PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 14/435, 14/46, A61K 38/16, 49/00, C12N 1/38, G01N 33/50

A1

(11) International Publication Number: WO 95/13291

(43) International Publication Date: 18 May 1995 (18.05.95)

(21) International Application Number: PCT/US94/12858

(22) International Filing Date: 8 November 1994 (08.11.94)

(30) Priority Data:
08/149,188 8 November 1993 (08.11.93) US

(71) Applicant: NEW YORK UNIVERSITY [US/US]; 70 Washington Square North, New York, NY 10012 (US).

(72) Inventor: GRUMET, Martin; Apartment 4F, 55 East 87th Street, New York, NY 10128 (US).

(74) Agents: LIVNAT, Shmuel et al.; Morrison & Foerster, 2000 Pennsylvania Avenue, N.W., Washington, DC 20006 (US).

(81) Designated States: AU, CA, JP.

Published
With international search report.

(54) Title: NEURON-GLIA CELL ADHESION MOLECULE, NG-CAM, IN TREATMENT OF NERVE DAMAGE

(57) Abstract

Neuron-glia cell adhesion molecule (Ng-CAM), alone or in combination with one or more additional agents, is useful in promoting the regeneration of a nerve in a subject having peripheral or spinal nerve damage. Pharmaceutical compositions comprising Ng-CAM are disclosed. Also provided are methods for diagnosing a neuronal disorder associated with abnormal levels of Ng-CAM and methods for assaying a test agent for its ability to enhance or inhibit the activity of Ng-CAM in promoting nerve regeneration.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

NEURON-GLIA CELL ADHESION MOLECULE, NG-CAM, IN TREATMENT OF NERVE DAMAGE

This invention was funded in part by a research grant from the National Institutes of Health, which provides to the United States Government certain rights in this invention.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention in the field of neuroscience and medicine relates to methods for promoting neurite growth and for promoting the growth and regeneration of nerves and treating spinal cord injury using neuron-glia cell adhesion molecule (Ng)-CAM or a functional derivative thereof.

Description of the Background Art

It has been estimated that more than 200,000 nerve repair procedures are performed annually in the United States. Unfortunately, the outcome of current peripheral nerve repair is generally poor (Archibald, S.J. *et al.*, 1991, *J. Comp. Neurol 306*:685-696). The limited regrowth of nerve fibers in the mature nervous system of higher vertebrates is due largely to an inability of the axons to elongate in this environment rather to an intrinsic inability of the neurons to regrow. Strategies to improve nerve regrowth using permissive guides such as

peripheral nerve grafts and silicone tubes have resulted in some, but limited, regrowth (Archibald *et al., supra;* LeBeau, J.M. *et al.*, 1988, *J. Neurocytol. 17*:161-172).

Current strategies to repair peripheral nerve transection are directed to suturing of the proximal nerve to the distal stump with microsurgical techniques so that the regenerating fibers can course through the degenerating distal stump to reinnervate the original target. This is effective only for simple transection but not in the majority of cases where gaps are generated between the proximal fibers and the distal nerve stumps. When gaps of several millimeters occur, nerve regeneration is very poor. Attempts have been made to bridge the gaps using nerve autografts. As stated above, silicone tubes and collagen-based conduits are as, or more, effective in promoting regeneration in rodents and nonhuman primates (Archibald *et al., supra;* Gibson, K.L. *et al.*, 1989, *Microsurgery 10*: 126-129). Gibson *et al.* discloses the implantation of nerve cuffs or guide tubes ("entubulization") as a method for repair of transected nerves. In this approach, proximal and distal nerve stumps are introduced into each end of a tube and are held together by one or two epineurial sutures. The regenerating axons travel through the lumen of the guide tube toward the distal stump. Several types of materials are known to serve as nerve cuffs, including natural materials including veins and autogenous collagen, and synthetic substances including lactate polymers, polygalactin mesh, polyethylene and silicone tubing.

The entubulation techniques open new possibilities for improving regeneration by incorporating various materials. Much has recently been learned about proteins

normally expressed abundantly, but transiently, during nerve development which are re-expressed during nerve regeneration in animals. The present inventor has turned his attention to the use of such neurally active proteins in promoting nerve repair and regrowth.

Cell Adhesion Molecules and Ng-CAM

Cell-cell adhesion is a primary process that is critical for embryonic development and for pattern formation in the nervous system (Edelman, G.M. *et al.*, 1990, *MORPHOREGULATORY MOLECULES*, John Wiley & Sons, New York). The ability of neurons to organize into specific patterns depends on their interactions with other neurons, with glia, and with the extracellular environment (Jacobson, M. ,1991, *DEVELOPMENTAL NEUROBIOLOGY*, 3rd Edition, Plenum Press, New York). Many of these interactions between neural cells are mediated by cell adhesion molecules (CAMs) (Edelman, G.M., 1983, *Science 219*:450-457) which fall primarily into two different families. Members of the immunoglobulin superfamily (Edelman, G.M., 1987, *Immun. Rev. 100*:11-45) contain immunoglobulin domains and fibronectin type III repeats and have calcium-independent binding, while members of the cadherin family share distinct repeated domains and have calcium-dependent binding (Takeichi, M., 1988, *Development 102*:639-655).

CAMs such as the neuron-glia CAM, Ng-CAM (Grumet, M. *et al.*, 1984, *J Cell Biol 98*:1746-1756) mediate cell-cell interactions and are expressed early during development in spatially and temporally restricted patterns (Edelman, G.M., 1988, *Biochemistry 27*:3533-3543; Jessell, T.M., 1988, *Cell 1*:3-13). Certain CAMs are transmembrane proteins which may participate in the transmission of signals between cells to modulate cell behavior and

differentiation (Edelman G.M., 1976, *Science 192*:218-226; Schuch, U. *et al.*, 1989, *Neuron 3*:13-20; Bixby, J.L., 1989, *Neuron 3*:287-297).

Ng-CAM is a large neuronal CAM of around 200 kDa that can mediate neuron-neuron and neuron-glia adhesion, and has been implicated in neuronal migration and the formation of nerve bundles. The biochemistry and biology of Ng-CAM is reviewed in Grumet, M., 1992, *J. Neurosci. Res. 31*:1-13, which is hereby incorporated by reference in its entirety. The biochemical properties of Ng-CAM are described below. Purified Ng-CAM presented as a substrate for neurons in culture can promote neuritic fiber extension of about 100 μm in several hours. Ng-CAM binds homophilically (to itself) and heterophilically to several cell surface proteins. It is structurally related to a human protein called L1 (Reid, R.A. *et al.*, 1992, *J. Mol. Neurosci. 3*:127-135), and it can bind to mammalian L1 (Grumet, M. *et al.*, 1986, *J. Cell Biol. 106*:487-503; Lemmon, V. *et al.*, 1989, *Neuron 2*:1597-1603). The binding of certain adhesion molecules, including L1, to neurons generates signals such as an increase in intracellular calcium that have been associated with promotion of neurite growth (Schuch *et al.*, *supra*; Williams, E.J. *et al.*, 1992, *J. Cell Biol. 119*:883-892). Thus, these proteins which have been implicated as major mediators of axonal growth (a) are expressed at high levels at times and locations of nerve formation during development, (b) decrease their expression during maturation of the nervous system, (c) dramatically increase expression following injury to the nervous system and (d) return to normal levels after recovery from injury (Daniloff, J.K. *et al.*, 1986b, *J. Cell. Biol. 103*:929-945)).

Ng-CAM shares certain properties with the neural CAM, N-CAM, which was the first CAM isolated from brain (Hoffman, S. *et al.*, 1982, *J. Biol. Chem. 257*:7720-7729). Unlike Ng-CAM which is specific to the nervous system, N-CAM is found in muscle and other tissues. N-CAM is a large cell surface glycoprotein which binds homophilically to N-CAM on other cells (Hoffman, S. *et al.*, 1983, *Proc Natl Acad Sci USA 80*:5762-5766) and to heparin by a different mechanism (Cole, G.J. *et al.*, 1989, *Neuron 2*:1157-1165). N-CAM has a role in cell adhesion (Brackenbury *et al.*, 1977, *J. Biol. Chem. 252*:6835-6840) retinal layering (Buskirk *et al.*, 1980, *Nature 285*:488-489), retinotectal mapping (Fraser *et al.*, 1984, *Proc. Natl. Acad. Sci. USA 81*:4222-4226) and neuron-myotube interaction (Rutishauser *et al.*, *J. Cell Biol. 97*:145-152 1983). N-CAM, however, does not play a major role in adhesion between embryonic neurons and glia in as much as most glial cells express low levels of N-CAM (Grumet *et al.*, 1983, *Science 222*:60-62). Although both N-CAM and Ng-CAM are involved in neuron-neuron adhesion and are members of the immunoglobulin superfamily, they are distinct molecules (Cunningham, B.A. *et al.*, 1987, *Science 236*:799-806; Burgoon, M.P. *et al.*, 1991, *J. Cell Biol. 112*:1017-1029). Chick proteins isolated independently and named by others (*e.g.*, G4 (Rathjen, F.G. *et al.*, 1987a, *J. Cell Biol. 104*:343-353) and 8D9 antigen (Lemmon, V. *et al.*, 1986, *J. Neurosci. 6*:2987-2994) are probably identical to Ng-CAM (Burgoon *et al.*, *supra*).

Following peripheral nerve injury, the amount of N-CAM and Ng-CAM increased dramatically in the region surrounding the site of injury, in the proximal nerve, and in the dorsal root ganglia while Ng-CAM levels

decreased moderately in the spinal cord. The levels of Ng-CAM returned to normal after nerve regeneration. It was pointed out that such correlations may have practical applications in studying nerve repair (Daniloff *et al.*, *supra*). Remsen, L.G. *et al.*, 1990, *Exper. Neurol.* *110*:268-273, showed that when tubes applied to transected sciatic nerves contained monoclonal antibodies (mAbs) specific to N-CAM, functional neuronal recovery was inhibited, indicating a role for N-CAM in nerve regeneration. Daniloff, U.S. Patent No. 4,955,892 (11 Sept 1990) discloses use of N-CAM in nerve prostheses for repair of peripheral nerve damage and restitution of muscle innervated by the regenerated nerve. This reference has no disclosure of Ng-CAM in such a therapeutic setting.

Characterization of Ng-CAM Protein

Monoclonal antibodies specific for Ng-CAM recognize a major component having a relative MW of 135 kDa and lesser amounts of two closely spaced components at about 200 kDa in extracts from chick brain (Grumet *et al.*, 1984a, *Proc Natl Acad Sci USA 81*:7989-7993). SDS-PAGE and protein staining of immunoaffinity purified Ng-CAM reveals an additional component of about 80 kDa which is not directly recognized by the anti-Ng-CAM mAbs. Rabbit antibodies were raised against each of the above three fractions. Antibodies against the 200 kDa protein recognized all three components. Antibodies against the 135 kDa protein recognized the 200 kDa but not the 80 kDa component. Antibodies against the 80 kDa component recognized the 200 kDa but not the 135 kDa protein (Grumet *et al.*, 1984a, *supra*; Wolff, J.M. *et al.*, 1987, *Eur. J. Biochem. 168*:551-561). Thus, each of the smaller components is antigenically related to the larger one but

not to each other, indicating possible cleavage of the 200 kDa component to yield the two smaller polypeptides. Pulse-chase biosynthesis experiments indicated that Ng-CAM is first synthesized as a 200 kDa species with the 135 kDa appearing later. The 200 kDa and 80 kDa components were difficult to analyze directly because of their relative instability even in the presence of protease inhibitors (Grumet *et al.*, 1988, *J Cell Biol 106*:487-503). Nevertheless, peptide mapping showed that the two smaller components of Ng-CAM share fragmentation patterns with the larger but not with each other (Wolff *et al.*, *supra*). The amino terminal sequences of Ng-CAM polypeptides are identical for the two components at ~200 kDa (*i.e.*, 210 and 190 kDa) and the 135 kDa component, and these differ from the amino terminal sequence of the 80 kDa species (Burgoon *et al.*, *supra*).

The findings regarding the protein structure of Ng-CAM are summarized in the form of a linear model (Figure 10) of its components which include amino terminal sequence similarities, immunological relationships, and structural relationships among the various components. Moreover, the sequence of Ng-CAM cDNA and analysis of its mRNA support this model.

SUMMARY OF THE INVENTION

The present inventor has conceived of the use of Ng-CAM or mammalian L1 or NILE protein, or functional derivatives thereof such as peptides representing regions of the full protein, to promote neurite sprouting and nerve growth. These proteins or derivatives are therefore useful in promoting nerve regeneration and

repair, for example, in the treatment of spinal cord injuries. These cell adhesion molecules may contribute to nerve regeneration and neuronal repair by: (1) promoting neuronal adhesion, (2) neutralizing inhibitory effects of extracellular matrix molecules such as chondroitin sulfate proteoglycans (*e.g.*, neurocan and 3F8 proteoglycan (PG)), and (3) providing stimulatory signals to neurons that alter intracellular messengers to promote neurite growth.

Ng-CAM/L1 may be particularly effective in promoting nerve regrowth in peripheral nerve injuries as well as in lesions in the central nervous system (CNS).

The present invention is thus directed to a method of promoting the regeneration of a nerve in a subject having peripheral or central nerve damage, comprising administering to a subject in need of such treatment an amount of Ng-CAM, or a functional derivative thereof, or cells expressing Ng-CAM, effective in promoting regeneration of nerves.

In another embodiment of the above method, Ng-CAM is administered in combination with an effective amount of at least one other agent that is capable of promoting neuronal survival, growth, differentiation or regeneration. Preferably the other agent is nerve growth factor, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), or another neurotrophin including NT-3, NT-4 or NT-5, or laminin.

The above methods are useful in treating peripheral nerve damage associated with physical or surgical trauma, infarction, bacterial or viral infection, toxin exposure, degenerative disease or malignant disease that affects peripheral or central neurons. Such disease further

include CNS lesions, gliosis, Parkinson's disease, Alzheimer's disease, neuronal degeneration, and the like. The present methods are useful for treating any disorder which induces a gliotic response or inflammation.

In the above methods, Ng-CAM or its functional derivative may be administered in a form associated with a solid or semisolid phase support material, preferably collagen gel.

The present invention is further directed to a pharmaceutical composition useful in the treatment of peripheral or central nerve damage, comprising:

(a) an amount of Ng-CAM effective for treating peripheral nerve damage; and

(b) a pharmaceutically acceptable carrier.

The pharmaceutical composition may also comprise at least one other agent that is capable of promoting neuron survival, growth, differentiation or regeneration or a material such as collagen gel which serves as a substrate for nerve growth.

The present invention also provides a method of promoting regeneration of a injured or severed nerve, comprising exposing an injured or severed nerve to a concentration of Ng-CAM that is effective in promoting the regeneration of neurons. This method may be carried out *in vitro* or *in vivo*.

Also provided is a method for promoting the regeneration of a severed nerve in a subject, comprising surgically entubulating the nerve in an entubulation device which contains an amount of Ng-CAM effective in promoting the regeneration. The device may further contain at least one other agent that is capable of promoting neuron survival, growth, differentiation or regeneration.

In another embodiment, the present invention is directed to a method for promoting neuronal survival or neurite growth by neutralizing or overcoming the inhibitory effect of a chondroitin sulfate proteoglycan, preferably neurocan, on the survival or growth, which method comprises contacting a nerve fiber inhibited in its survival or growth by a chondroitin sulfate proteoglycan with an amount of Ng-CAM effective in neutralizing or overcoming the inhibitory effect, thereby promoting neuronal survival or neurite growth.

The present invention is further directed to a method of diagnosing a neuronal disorder associated with an abnormal level of a substance which binds to Ng-CAM in a subject, comprising:

(a) measuring the level of the Ng-CAM-binding substance in a sample from the subject; and

(b) comparing the levels of the substance measured in step (a) with the level of the substance in an analogous sample from a normal individual or a standard level of the substance,

thereby detecting an abnormality in the level of the Ng-CAM-binding substance in the subject, the abnormality being diagnostic of the neuronal disorder. The Ng-CAM-binding substance is preferably a chondroitin sulfate proteoglycan, such as the 3F8 proteoglycan.

Also provided is a method for identifying a compound or agent which binds to Ng-CAM or to a functional derivative thereof, comprising:

(a) exposing said compound or agent to Ng-CAM or a functional derivative thereof, preferably bound to a solid phase carrier or support;

(b) measuring the binding of the compound or agent to the Ng-CAM or functional derivative.

The present invention also provides a method of diagnosing a neuronal disorder, such as a brain tumor, associated with an abnormal level of Ng-CAM in a subject, comprising:

(a) measuring the level of Ng-CAM in a sample from the subject; and

(b) comparing the levels of Ng-CAM measured in step (a) with the level of Ng-CAM in an analogous sample from a normal individual or a standard level,

thereby detecting an abnormality in the level of Ng-CAM in the subject, the abnormality being diagnostic of the neuronal disorder.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing binding of neurocan and aggrecan to CAMs and extracellular matrix proteins. Wells of removable 96-well plates were coated with unlabeled proteins (1.25 μg/ml) and incubated with ^{125}I-labeled proteins (~160,000 cpm). The fraction of the input bound to the different substrates is given as a percentage. Nonspecific binding to BSA was also determined, and specific binding (percent bound) was calculated as total minus nonspecific binding. Specific activities in the experiments shown here were 2.5 to 2.9 x 10^{18} cpm/mole. All values are means of duplicate determinations ± SEM.

Figure 2 is a graph showing binding of neurocan to CAMs and collagen I under hypotonic and isotonic conditions. Labeled proteoglycans were applied at an average of 160,000 cpm per well. All values are means of duplicate determinations ± SEM.

Figure 3 shows saturation curves (panels A and B) and Scatchard plot analysis (panels C and D) of ^{125}I-labeled 7-d neurocan (Panels A and C) and neurocan-C (panels B and D) binding to Ng-CAM. Binding values represent specific binding (total cpm bound minus cpm bound to BSA). Neurocan was tested at 0.5-50 ng/well (6.8 x 10^{18} cpm/mole), and neurocan-C at 0.2-37 ng/well (4.8 x 10^{18} cpm/mole). Bars in the saturation curves represent the SEM of duplicate determinations.

Figure 4 is a series of graphs showing saturation curves (panels A and B) and Scatchard analysis (panels C and D) of ^{125}I-labeled neurocan (panels A and C) and neurocan-C (panels B and D) binding to N-CAM. Binding values represent specific binding (total cpm bound - cpm bound to BSA). Neurocan was tested at 0.4-40 ng/well and neurocan-C at 1-60 ng/well. Bars in saturation curves represent the SEM of duplicate determinations.

Figure 5 is a graph showing the binding of ^{125}I-labeled neurocan-C to Ng-CAM in the presence of other soluble molecules. Wells coated with Ng-CAM were incubated with ^{125}I-labeled neurocan-C (160,000 cpm/well) in the presence of unlabeled neurocan-C (●), aggrecan (◆), chondroitin sulfate (◇), chondroitin sulfate disaccharides (▲), and fibronectin (O) at the concentrations indicated. Specific binding of neurocan-C to Ng-CAM in the absence of soluble molecules corresponds to 0% inhibition; background binding to BSA corresponds to 100% inhibition. Values are the means of duplicate determinations and the SEM is less than 5% of the mean value.

Figure 6 is a set of graphs showing effects of chondroitinase and heat treatment on the binding of neurocan to Ng-CAM (panel A) and to N-CAM (Panel B).

"native" - Native proteoglycans; "control" - proteoglycans incubated at 37°C for 2 h in chondroitinase buffer; "ch-ase" - chondroitinase-treated proteoglycans; "heat" - proteoglycans incubated at 95°C for 15 min; "ch-ase+heat" - chondroitinase and heat-treated proteoglycans. ^{125}I-labeled neurocan were used at an average of 55,000 cpm/well. All values are means of duplicate determinations ± SEM.

Figure 7 is a series of micrographs showing immunoperoxidase staining of 7-d postnatal rat brain cerebellum with antibodies to neurocan, Ng-CAM, and N-CAM. Panel A: mAb 2C2, which recognizes a cytoplasmic region that is highly conserved between avian Ng-CAM and mammalian NILE/L1; Panel B: mAb 1D1 specific for neurocan; Panel C: mAb 5B8 specific for N-CAM. Bar = 50 μm.

Figure 8 is a series of micrographs showing effects of neurocan on neurite growth on Ng-CAM and anti-Ng-CAM mAbs. Substrates were incubated first with 50 μg/ml Ng-CAM (Panels A and B) or 3 μg/ml mAb anti Ng-CAM IgG (Panels C and D), followed by incubation with either 30 μg BSA (A and C) or neurocan (B and D). Brain cells from 9-d chick embryos were added to the substrates, fixed after 2 days in culture and photographed under phase microscopy. Bar = 50 μm.

Figure 9 is a series of graphs showing effects of neurocan on neurite outgrowth. Brain cells from 9-d chick embryos were plated on substrates coated with 50 μg/ml Ng-CAM (Panel A) or 3 μg/ml mAb anti Ng-CAM IgG (Panel B), and either 30 μg BSA or 11 μg neurocan. After 2 days in culture, cells were fixed and, for each bound cell, the length of the longest neurite was determined. The histograms show the percentage of

neurites having the designated lengths. The following were the values of mean neurite lengths in μM ± SEM:

Ng-CAM minus neurocan: 19.3 ± 1.8 (n = 148);
Ng-CAM plus neurocan: 6.7 ± 1.1 (n = 66);
anti Ng-CAM minus neurocan: 19.4 ± 1.7 (n = 89);
anti Ng-CAM plus neurocan: 6.3 ± 0.9 (n = 152).

Figure 10 shows a model of Ng-CAM protein and domain structure. (a) Linear representation of Ng-CAM components aligned to indicate relationships between them. The 135 kDa component, which is the predominant form found in the chick, and the 80 kDa component are derived from the 200 kDa by proteolysis. The general locations of N-linked carbohydrates (CHO), phosphorylated amino acids (P), and covalently bound fatty acids (swiggled line) are indicated. (b) The six immunoglobulin domains (each ~100 amino acids) are represented by loops near the amino terminus (NH2) and the fibronectin type III repeats (each ~100 amino acids) are represented by the rectangles. The two last repeats (shown as open rectangles) have much lower identities to fibronectin type III repeats. The transmembrane domain (23 amino acids) is indicated by the vertical stippled bar, and the cytoplasmic region (113 amino acids) is near the carboxy terminus (COOH). The location of the amino terminus of the 80 kDa component is indicated by an arrow.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Ng-CAM/L1 binds homophilically to other Ng-CAM molecules and heterophilically to several cell surface proteins that are found in the nervous system. The

binding of certain adhesion molecules (including L1) to neurons generates intracellular signals (such as increase in calcium) associated with promotion of neurite growth. The present inventor recognized that the ability of Ng-CAM/L1 to provide signals that promote neurite growth as well as to serve directly as favorable substrates for neuronal adhesion and migration, make it an excellent candidate for use in (1) improving nerve regeneration or promoting nerve survival, (2) treatment of peripheral nerve injury and spinal cord injury and (3) stimulation of growth of endogenous, implanted or transplanted CNS tissue.

Because Ng-CAM normally reappears during regeneration in a delayed manner (Daniloff *et al.* 1986, *supra*), introduction of Ng-CAM or a derivative thereof soon after injury is particularly important for accelerating the rate and the extent of recovery.

Cell repulsion or avoidance is a process that occurs during normal neural development and may be important for regeneration. Nerves will not grow into regions myelinated by oligodendrocytes; certain proteins are believed to be responsible for this repulsion. Chondroitin sulfate proteoglycans have repulsive effects on neuronal adhesion and fiber growth. The present inventor and his collaborators have recently shown that two chondroitin sulfate proteoglycans from brain, neurocan and 3F8 (Rauch, U. *et al.*, 1991, *J. Biol. Chem. 266*:14785-14801) inhibit Ng-CAM function and bind to Ng-CAM with high affinity (Grumet, M. *et al.*, 1993, *J. Cell. Biol 120*:815-824; Friedlander, D.R. *et al.*, 1993, *J. Neurosci. 19*:626a). These proteoglycans may therefore inhibit nerve regrowth and neuronal cell division. According to the present invention, Ng-CAM is useful to

neutralize or counteract their inhibitory properties on neurons.

In particular, the present inventor and his coworkers have found that neurocan and 3F8 proteoglycan, chondroitin sulfate proteoglycans of brain, in soluble form, bind with high affinity to Ng-CAM. Neurocan-mediated inhibition of neuronal adhesion was related to blockage of binding to substrate-bound Ng-CAM in *in vitro* assays. Longer term assays showed that neurocan inhibited neurite outgrowth on Ng-CAM substrates under conditions similar to those that inhibited neuronal adhesions. Moreover, Ng-CAM reversed the inhibition by substrate-bound neurocan and stimulated both neuronal adhersion and neurite growth.

On the basis of these observations, the present inventor conceived of the use of Ng-CAM to promote neural recovery from injury, such as spinal cord injury, by neutralizing the inhibitory action of proteins such as neurocan on the repair process.

The ability of Ng-CAM/L1 to provide signals that favor neurite growth as well as to serve directly as favorable substrates for adhesion and migration, make it an excellent candidate for use to improve nerve regeneration. Although these molecules normally reappear during regeneration, they do so in a delayed manner in the peripheral nervous system. Therefore, their introduction soon after injury may be particularly important for accelerating the rate and the extent of the recovery.

Ng-CAM/L1 PROTEINS, PEPTIDES AND THEIR FUNCTIONAL DERIVATIVES

It will be understood that the Ng-CAM/L1 protein useful in the methods and compositions of the present invention can be biochemically purified from a cell or tissue source. For preparation of naturally occurring Ng-CAM, tissues such as brain, especially of human origin, are preferred.

Alternatively, because the gene encoding Ng-CAM or L1 can be isolated or synthesized, the polypeptide can be synthesized substantially free of other proteins or glycoproteins of mammalian origin in a prokaryotic organism or in a non-mammalian eukaryotic organism, if desired.

Alternatively, methods are well known for the synthesis of polypeptides of desired sequence on solid phase supports and their subsequent separation from the support.

In a further embodiment, the invention provides "functional derivatives" of Ng-CAM/L1. By "functional derivative" is meant a "fragment," "variant," "analog," or "chemical derivative" of the Ng-CAM/L1. A functional derivative retains at least a portion of the function of Ng-CAM, such as binding to Ng-CAM, binding to neurocan, binding to a specific anti-Ng-CAM antibody, or stimulation of neurite growth, which permits its utility in accordance with the present invention.

A "fragment" of Ng-CAM/L1 protein refers to any subset of the molecule, that is, a shorter peptide.

A "variant" of Ng-CAM/L1 refers to a molecule substantially similar to either the entire protein or a fragment thereof. Variant peptides may be conveniently prepared by direct chemical synthesis of the variant peptide, using methods well- known in the art.

Alternatively, amino acid sequence variants of the protein or peptide can be prepared by mutations in the DNA which encodes the synthesized peptide. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence. Any combination of deletion, insertion, and substitution may also be made to arrive at the final construct, provided that the final construct possesses the desired functional activity. Obviously, the mutations that will be made in the DNA encoding the variant peptide must not alter the reading frame and preferably will not create complementary regions that could produce secondary mRNA structure (see European Patent Publication No. EP 75,444).

At the genetic level, these variants ordinarily are prepared by site-directed mutagenesis (as exemplified by Adelman *et al.*, *DNA* 2:183 (1983)) of nucleotides in the DNA encoding the Ng-CAM/L1 protein or a peptide fragment thereof, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture (see below). The variants typically exhibit the same qualitative biological activity as the nonvariant peptide.

A preferred group of variants of Ng-CAM/L1 are those in which at least one amino acid residue in the protein or in a peptide fragment thereof, and preferably, only one, has been removed and a different residue inserted in its place. For a detailed description of protein chemistry and structure, see Schulz, G.E. *et al.*, *PRINCIPLES OF PROTEIN STRUCTURE*, Springer-Verlag, New York, 1978, and Creighton, T.E., *PROTEINS: STRUCTURE AND MOLECULAR PROPERTIES*, W.H. Freeman & Co., San Francisco, 1983, which are hereby incorporated by

reference. The types of substitutions which may be made in the protein or peptide molecule of the present invention may be based on analysis of the frequencies of amino acid changes between a homologous protein of different species, such as those presented in Table 1-2 of Schulz *et al.* (*supra*) and Figure 3-9 of Creighton (*supra*). Base on such an analysis, conservative substitutions are defined herein as exchanges within one of the following five groups:

1. Small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr (Pro, Gly);
2. Polar, negatively charged residues and their amides: Asp, Asn, Glu, Gln;
3. Polar, positively charged residues: His, Arg, Lys;
4. Large aliphatic, nonpolar residues: Met, Leu, Ile, Val (Cys); and
5. Large aromatic residues: Phe, Tyr, Trp.

The three amino acid residues in parentheses above have special roles in protein architecture. Gly is the only residue lacking any side chain and thus imparts flexibility to the chain. Pro, because of its unusual geometry, tightly constrains the chain. Cys can participate in disulfide bond formation which is important in protein folding. Note the Schulz *et al.* would merge Groups 1 and 2, above. Note also that Tyr, because of its hydrogen bonding potential, has some kinship with Ser, Thr, etc. Substantial changes in functional or immunological properties are made by selecting substitutions that are less conservative, such as between, rather than within, the above five groups, which will differ more significantly in their effect on maintaining (a) the structure of the peptide backbone in

the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Examples of such substitutions are (a) substitution of gly and/or pro by another amino acid or deletion or insertion of gly or pro; (b) substitution of a hydrophilic residue, e.g., ser or thr, for (or by) a hydrophobic residue, e.g., leu, ile, phe, val or ala; (c) substitution of a cys residue for (or by) any other residue; (d) substitution of a residue having an electropositive side chain, e.g., lys, arg or his, for (or by) a residue having an electronegative charge, e.g., glu or asp; or (e) substitution of a residue having a bulky side chain, e.g., phe, for (or by) a residue not having such a side chain, e.g., gly.

Most deletions and insertions, and substitutions according to the present invention are those which do not produce radical changes in the characteristics of the protein or peptide molecule. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays which are described in more detail below. For example, a change in the immunological character of the protein peptide molecule, such as binding to a given antibody, is measured by a competitive type immunoassay. Biological activity is screened in an appropriate bioassay, as described below.

Modifications of such peptide properties as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation or the tendency to aggregate with

carriers or into multimers are assayed by methods well known to the ordinarily skilled artisan.

An "analog" of Ng-CAM/L1 refers to a non-natural molecule substantially similar to either the entire molecule or a fragment thereof.

A "chemical derivative" of Ng-CAM/L1 contains additional chemical moieties not normally a part of the peptide. Covalent modifications of the peptide are included within the scope of this invention. Such modifications may be introduced into the molecule by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues.

Additionally, modified amino acids or chemical derivatives of amino acids of Ng-CAM/L1 or fragments thereof, according to the present invention may be provided, which polypeptides contain additional chemical moieties or modified amino acids not normally a part of the protein. Covalent modifications of the peptide are thus included within the scope of the present invention. The following examples of chemical derivatives are provided by way of illustration and not by way of limitation.

Aromatic amino acids may be replaced with D- or L-naphthylalanine, D- or L-phenylglycine, D- or L-2-thienylalanine, D- or L-1-, 2-, 3- or 4-pyrenyl-alanine, D- or L-3-thienylalanine, D- or L-(2-pyridinyl)-alanine, D- or L-(3-pyridinyl)-alanine, D- or L-(2-pyrazinyl)-alanine, D- or L-(4-isopropyl)-phenylglycine, D-(trifluoromethyl)-phenylglycine, D-(trifluoromethyl)-phenylalanine, D-p-fluorophenylalanine, D- or L-p-biphenylphenylalanine, D- or L-p-methoxy-biphenylphenylalanine, D- or L-2-indole(alkyl)alanine,

and D- or L-alkylalanine where alkyl may be substituted or unsubstituted methyl, ethyl, propyl, hexyl, butyl, pentyl, iso-propyl, iso-butyl, sec-isotyl, iso-pentyl, non-acidic amino acids, of C1-C20.

Acidic amino acids can be substituted with non-carboxylate amino acids while maintaining a negative charge, and derivatives or analogs thereof, such as the non-limiting examples of (phosphono)-alanine, glycine, leucine, isoleucine, threonine, or serine; or sulfated (for example, $-SO_3H$) threonine, serine, tyrosine.

Other substitutions may include unnatural hydroxylated amino acids may made by combining "alkyl" (as defined and exemplified herein) with any natural amino acid. Basic amino acids may be substituted with alkyl groups at any position of the naturally occurring amino acids lysine, arginine, ornithine, citrulline, or (guanidino)-acetic acid, or other (guanidino)alkyl-acetic acids, where "alkyl" is define as above. Nitrile derivatives (e.g., containing the CN-moiety in place of COOH) may also be substituted for asparagine or glutamine, and methionine sulfoxide may be substituted for methionine. Methods of preparation of such peptide derivatives are well known to one skilled in the art.

In addition, any amide linkage in any of neurocan polypeptides can be replaced by a ketomethylene moiety, *e.g.*, $(-C(=O)-CH_2-)$ for $(-(C=O)-NH-)$. Such derivatives are expected to have the property of increased stability to degradation by enzymes, and therefore possess advantages for the formulation of compounds which may have increased *in vivo* half lives, as administered by oral, intravenous, intramuscular, intraperitoneal, topical, rectal, intraocular, or other routes.

In addition, any amino acid representing a component of the said peptides can be replaced by the same amino acid but of the opposite chirality. Thus, any amino acid naturally occurring in the L-configuration (which may also be referred to as the R or S, depending upon the structure of the chemical entity) may be replaced with an amino acid of the same chemical structural type, but of the opposite chirality, generally referred to as the D-amino acid but which can additionally be referred to as the R- or the S-, depending upon its composition and chemical configuration. Such derivatives have the property of greatly increased stability to degradation by enzymes, and therefore are advantageous in the formulation of compounds which may have longer *in vivo* half lives, when administered by various routes.

Additional amino acid modifications of amino acids of a Ng-CAM/L1/NILE protein or peptide according to the present invention may include the following.

Cysteinyl residues most commonly are reacted with alpha-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, alpha-bromo- beta-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4- nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylprocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is

preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing alpha-amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3- butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues per se has been studied extensively, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizol and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R'-N-C-N-R') such as 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide or 1- ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl

residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Derivatization with bifunctional agents is useful for cross-linking the peptide to a water-insoluble support matrix or to other macromolecular carriers. Commonly used cross-linking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidyl-propionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains (Creighton, *supra*), acetylation of the N-terminal amine, and, in some instances, amidation of the C-terminal carboxyl groups.

Such derivatized moieties may improve the solubility, absorption, biological half life, and the like. The moieties may alternatively eliminate or attenuate any undesirable side effect of the protein and the like. Moieties capable of mediating such effects are disclosed, for example, in *Remington's Pharmaceutical Sciences*, 16th ed., Mack Publishing Co., Easton, PA (1980)

PRODUCTION OF CHICKEN NG-CAM AND HUMAN L1 FUSION PROTEINS THAT PROMOTE NEURITE GROWTH

Fusion proteins representing different polypeptide regions in Ng-CAM or L1 are used to identify regions of Ng-CAM and human L1 that have the desired functional activity (binding, stimulating neurite growth, etc.). When combined with the PCR, it is thus possible and expedient to express in bacteria nearly any selected region of the protein.

To facilitate unidirectional subcloning of the PCR products, sense and antisense oligonucleotides have been designed to include BamH1 recognition sequences at the 5' end and EcoR1 recognition sequences at the 3' end, respectively; appropriately digested PCR products are then be ligated directly into a vector (such as the pGEX-2T vector).

Use of this methodology allows construction of vectors and purification of several fusion proteins in less than one month.

The pGEX vector is preferred chosen because the glutathione-S-transferase (GST) fusion proteins can be purified rapidly by binding to glutathione-agarose beads. In addition, because cDNAs are cloned into pGEX-2T, the portion of the fusion protein representing the GST can be cleaved with thrombin and the engineered polypeptide can

generally be recovered free of the GST protein which can be removed using glutathione-agarose beads (Ausubel, F.M., *et al.*, 1990, *CURRENT PROTOCOLS IN MOLECULAR BIOLOGY*, John Wiley & Sons, New York.

Ng-CAM or fusion proteins thereof may also be expressed in insect cells using baculovirus expression system. Production of Ng-CAM or functional derivatives thereof, including fusion proteins, in insects can be achieved, for example, by infecting the insect host with a baculovirus engineered to express Ng-CAM by methods known to those of skill. Thus, in one embodiment, sequences encoding Ng-CAM may be operably linked to the regulatory regions of the viral polyhedrin protein (Jasny, 1987, *Science 238*:1653). Infected with the recombinant baculovirus, cultured insect cells, or the live insects themselves, can produce the Ng-CAM or functional derivative protein in amounts as great as 20 to 50% of total protein production. When live insects are to be used, caterpillars are presently preferred hosts for large scale production according to the invention.

Fragments of Ng-CAM are purified by conventional affinity chromatography using monoclonal antibodies that recognize the appropriate regions of Ng-CAM.

Several constructs have been purified from existing cDNA clones (Burgoon *et al.*, *supra*) that represent different portions of the Ng-CAM ectodomain. The purified fusion proteins have been tested for binding to Ng-CAM, and for their ability to promote growth of neurites in culture. The results suggest that regions in the first two or three Ig-like domain mediate homophilic Ng-CAM binding and can promote neurite growth (Figure 10).

In addition, other regions such as in the fifth and sixth Ig-like domains do not bind to Ng-CAM but nevertheless promote neurite growth. These regions may be involved in Ng-CAM binding to neurocan. The combined results suggest that different Ig-like domains in Ng-CAM may have different functions that are mediated by binding to different ligands.

Given the sequence similarities in the Ig-like domains between chicken Ng-CAM and human L1 (about 55% identity and >65% similarity when conservative amino acid substitutions are considered), it is likely that comparable regions in the molecules serve similar functions. To determine which Ig-like domains in L1 mediate neuronal binding and promote neurite growth, constructs representing different domains are prepared in pGEX. The choice of the regions to be expressed is initially guided by the functional properties of the existing Ng-CAM fusion proteins, where, for example, the first three Ig-like domains have been found to promote neurite growth. The fusion proteins will be expressed and characterized using established assays described herein, with neurons and L1 protein obtained from rats. Inasmuch as rat L1 is >92% identical in amino acid sequence to human L1, it is a suitable model for the human protein has not yet been isolated in sufficient quantities for these studies.

The mAbs specific for the most highly conserved regions in Ng-CAM can be used to purify L1 protein from rat brain; studies confirmed that rat L1 so isolated binds to Ng-CAM.

ASSAYS FOR PROTEINS AND PEPTIDES HAVING Ng-CAM-LIKE ACTIVITY

To characterize functions of different regions in Ng-CAM and L1, as well as peptides derived therefrom and other functional derivatives thereof, different assays for molecular binding, cell adhesion and neurite growth are used. These assays may be used routinely to analyze the biological functions of derivatives of Ng-CAM, such as peptide fragments. The combined use of these assays allows analysis of molecular mechanisms of binding as well as neurite growth.

Covasphere Assays:

Ng-CAM or fusion proteins thereof are covalently coupled to fluorescent beads and tested (1) for self-aggregation (measured using a Coulter Counter), and (2) for coaggregation with differently colored Covaspheres (observed by fluorescence microscopy and measured using a fluorescence activated cell sorter) and/or (3) for binding to cells expressing ligands for Ng-CAM ((Grumet *et al.*, 1988, *supra*; Grumet *et al.*, 1993, *supra*; Kuhn, T.B. *et al.*, *J. Cell Biol. 115*:1113-1126).

Radioligand Binding Assays

Proteins or peptides are labeled with ^{125}I and tested for binding to unlabeled proteins adsorbed to microwells of microplates (such as Immulon plate, Dynatech Labs). Such assays indicated that both ^{125}I-labeled Ng-CAM and ^{125}I-labeled neurocan bound to Ng-CAM with high affinity. Furthermore, cells may be transfected with DNA encoding a portion of the Ng-CAM molecule, representing about 60% of the extracellular region; such cells expressing this portion of Ng-CAM also bind ^{125}I-proteoglycan.

Gravity Cell Adhesion Assay

Proteins are adsorbed to 35 mm polystyrene petri dishes in 1 μl drops by incubation in a humid chamber; the unadsorbed proteins are removed and the remaining surface of the dishes is blocked with bovine serum albumin (BSA). Cells numbering from about 2 - 5 x 10^6 are incubated on the dishes for 1 h, the dishes are washed to remove unbound cells, and the binding of cells to different proteins is visualized by phase contrast microscopy. The number of cells bound is measured as described by Friedlander, D.R. *et al.* (1988), *J. Cell Biol. 107*:2329-2340; Grumet *et al.*, 1993, *supra*.

Neurite Growth Assay

Petri dishes are coated with proteins as described above for the gravity cell adhesion assay. Dissociated primary neurons from brain are prepared for culture by light trypsinization with EDTA as described by Grumet *et al.*, 1988, *supra*, and cultured in defined medium (DMEM plus ITS+).

Specific parameters of neurite growth which are recorded include the percentage of neurons with neurites, mean number of neurites per cell, and mean neurite length (Hoffman, S. *et al.*, 1986, *J. Cell Biol. 103*:145-158; Rogers, S.L. *et al.*, 1983, *Dev. Biol. 98*:212-220). As an estimate of neurite length, the distance between the cell body and the tip of the most distant neurite, the "neurite reach", is determined with an eyepiece micrometer.

cDNAs encoding human L1 (Reid *et al.*, *supra*) were obtained from Dr. John Hemperly. Both chicken and mammalian cells are tested for binding. Previous studies indicated that chicken Ng-CAM could bind to rodent L1.

The assays described above are used to functionally characterize engineered proteins representing regions of

L1. If, as expected, regions homologous to domains of Ng-CAM have similar activities, an analysis of their conserved sequences may provide additional clues for selecting particular amino acid sequences that are critical for binding in Ng-CAM and L1.

RELATIONSHIP BETWEEN BINDING DOMAINS IN AVIAN NG-CAM AND MAMMALIAN L1: IDENTIFICATION OF ACTIVE PEPTIDES

The mutual binding of avian Ng-CAM and mammalian L1 indicate that the amino acid sequences involved are conserved. Comparisons between amino acid sequences of Ng-CAM with L1 have revealed certain highly conserved regions. For example, although the overall amino acid identity between Ng-CAM and L1 in the first three Ig-like domains is only about 55% (somewhat higher if one allows for conservative substitutions), there are blocks of invariant sequence as opposed to those that are highly variable.

Using the assays described above, individual Ig-like domains and other peptide sequences will be examined for Ng-CAM-like function, such as induction of as neurite growth. This will define the amino acid sequences that mediate specific functions and will allow selection of peptides for therapeutic uses.

Thus, peptides of about 10-20 residues are synthesized and tested directly for:

(a) binding to L1;

(b) binding to neurocan;

(c) activity in promoting neuronal adhesion;

(d) activity in promoting neurite growth.

HETEROPHILIC BINDING OF Ng-CAM/L1:
INTERACTIONS WITH BRAIN PROTEOGLYCANS

In addition to homophilic binding, recent studies indicate that several molecules including neurocan and 3F8 proteoglycan may be heterophilic ligands for Ng-CAM/L1. Both neurocan, a chondroitin sulfate proteoglycan that is secreted by neurons, and 3F8 proteoglycan that is associated with astroglial cells, bind to Ng-CAM and inhibit homophilic Ng-CAM binding as well as neuronal adhesion and neurite growth to Ng-CAM in culture.

Rat neurocan and a second chondroitin sulfate proteoglycan, 3F8 (Rauch, U. *et al.*, 1991, *supra*), were obtained from Dr. Richard Margolis, New York University.

Ng-CAM/L1 binding sites for these proteoglycans will be identified for the preparation of peptides useful as potent neutralizers of inhibition of neuronal adhesion to Ng-CAM. Recent experiments by the present inventor and his colleagues have shown that ^{125}I-labeled neurocan and 3F8 proteoglycan bound to rat L1 and not to other adhesion proteins such as fibronectin and myelin associated glycoprotein. Both proteoglycans bound to Ng-CAM with high affinity ($K_d=1.3 \times 10^{-9}$ for neurocan, and $K_d=10^{-10}$ for 3F8 proteoglycan). The proteoglycans also bound to cells transfected to express about 60% of the extracellular portion of Ng-CAM.

The proteoglycans may act simply by blocking homophilic binding sites on cell adhesion molecules such as Ng-CAM. Alternatively, they may provide "inhibitory" signals to cells by binding to cell surface receptors. Both possibilities are of potential importance to neural regeneration because Ng-CAM polypeptides may directly promote neurite growth while at the same time they also

may neutralize "inhibitory" signals that may be generated by certain proteoglycans.

The radioligand and Covasphere assays described above will be used to test the binding of neurocan and 3F8 to fusion proteins, peptides and functional derivatives thereof, representing different regions of Ng-CAM/L1. Neurocan inhibits homophilic binding between purified Ng-CAM on Covasphere beads, suggesting that neurocan binds at or near the homophilic binding site, or alters the conformation of Ng-CAM by binding to a different site. The use of radioligand binding assays will indicate which domains in Ng-CAM/L1 are involved in homophilic binding and heterophilic binding to neurocan and 3F8 proteoglycans.

Peptides of varying lengths derived from Ng-CAM/L1 that bind to the proteoglycans with high affinity are useful for neutralizing the inhibitory effects of the proteoglycans on neuronal adhesion and neurite growth. Such peptides may be expressed in bacteria, or, more preferably, by a baculovirus expression system in insect cells. Such peptides are tested for their ability to bind to proteoglycans; peptides that can neutralize the inhibitory effects of the proteoglycans on neurite growth are useful as therapeutic agents.

THERAPEUTIC APPLICATIONS OF Ng-CAM/L1

The present invention provides for methods of treatment of a neuronal disorder, preferably a motor neuron disorder, in particular peripheral nerve damage or transection, which methods comprise administering to a subject in need of such treatment an effective amount of Ng-CAM/L1 or a functional derivative thereof, that

supports the survival, growth of the neurons and regeneration of the damaged nerve.

The disorders that may be treated according to this invention include, but are not limited to, disorders such as physical or surgical trauma, infarction, infection, toxin exposure, degenerative disease or malignant disease that affects peripheral or central neurons as well as any other components of the nervous system.

Effective doses of Ng-CAM/L1/NILE for therapeutic uses discussed above may be determined using methods known to one skilled in the art. Effective doses may be determined, preferably *in vitro*, in order to identify the optimal dose range using various of the methods described herein. In a preferred embodiment, an aqueous solution of a Ng-CAM/L1/NILE protein or peptide is administered by subcutaneous injection. Each dose may range from about 0.1 μg to about 100 μg/kg body weight, or more preferably, from about 1 μg to 50 μg/kg body weight. The dosing schedule may vary from once a week to daily depending on a number of clinical factors, including the type of disease, severity of disease, and the subject's sensitivity to the protein. Nonlimiting examples of dosing schedules are 3 μg/kg administered twice a week, three times a week or daily; a dose of 7 μg/kg twice a week, three times a week or daily; a dose of 10 μg/kg twice a week, three times a week or daily; or a dose of 30 μg/kg twice a week, three times a week or daily. In the case of more severe disease, it may be preferable to administer doses such as those described above by alternate routes, including intravenously or intrathecally. Continuous infusion may also be appropriate.

Ng-CAM/L1/NILE may also be administered via a cellular, solid or semi-solid implant to achieve blood levels of the protein or peptide similar to those attained by subcutaneous administration. A cellular implant may comprise cells naturally producing, or genetically altered to produce Ng-CAM/L1/NILE which secrete the protein or peptide *in vivo* in a subject following inoculation.

Ng-CAM/L1/NILE or a functional derivative may also be administered in combination with an effective amount of at least one other agent that is, itself, capable of promoting neuron survival, growth, or regeneration.

The Ng-CAM/L1/NILE may be administered in any pharmaceutically acceptable carrier. The administration route may be any mode of administration known in the art, including but not limited to intravenously, intrathecally, subcutaneously, or intracranially by injection into involved tissue, intraarterially, orally, or via an implanted device. Preferably, the Ng-CAM/L1 is added in combination with a nerve entubulation device or a gel, such as a collagen gel, to promote nerve regrowth.

The entubulation techniques, described above, for promoting nerve regeneration, can be combined with the use of proteins and peptides as described herein to promote nerve regeneration. Given their ability to promote neurite growth by various mechanisms, Ng-CAM/L1/NILE and functional derivatives thereof, are particularly useful for nerve regeneration when incorporated in synthetic entubulation devices.

For use in entubulation devices, Ng-CAM/L1/NILE or a functional derivative thereof in a dose ranging from about 0.01 μg/ml to about 2 mg/ml, more preferably, from about 1 μg/ml to 100 μg/ml.

In addition to the utility of Ng-CAM/L1 in the promotion of peripheral nerve regeneration, its mode of action indicates additional utilities. Ng-CAM/L1/NILE can act as a substrate for neurite growth, in the generation growth promoting signals in neurons, and as an agent capable neutralizing inhibitory effects of brain proteoglycans.

Thus, Ng-CAM/L1/NILE or functional derivatives thereof may also be used to treat other neural disorders which would benefit from increasing neurite sprouting and growth. In this regard, previous studies have shown that Ng-CAM can promote the formation and growth of complex growth cones which are a key hallmark of development and a vital aspect of regeneration. Introduction of polypeptides or peptides from Ng-CAM/L1 that promote growth may facilitate nerve regrowth in injuries to other neural regions such as the spinal cord and the brain where the potential for surgery is even more limited than in the peripheral nervous system.

Although direct introduction of polypeptides into the CNS is difficult and is limited by the blood brain barrier, penetration from the circulation does occur at sites of injury where the barrier is broken. Major efforts are currently under way to develop technologies to deliver proteins and polypeptides into the CNS. The present inventor's laboratory has found that Ng-CAM promotes outgrowth from explants of vertebrate nervous tissues, including brain, spinal cord and dorsal root ganglia. Thus, the present invention also includes the use of Ng-CAM or a functional derivative thereof to promote the growth and innervation by implanted neural tissues in a mammal.

In another embodiment, the Ng-CAM/L1/NILE or functional derivative can be impregnated into an implantable delivery device such as a cellulose bridge or sling prosthesis. Preferably, such a device is covered with glia, as described by Silver, *J. et al.*, 1983, *Science 220*:1067-1069 (1983), which reference is hereby incorporated by reference in its entirety. Thus, such a form of neuronal or axonal engineering can be used to rebuild a major nerve tract.

The present invention also provides pharmaceutical compositions comprising an amount of Ng-CAM/L1 or a functional derivative thereof effective to promote neuron growth or nerve regeneration, and effective to treat a disease associated with nerve damage or dysfunction, in a pharmaceutically acceptable carrier. Also provided is a pharmaceutical composition comprising an effective amount of Ng-CAM/L1 together with one or more additional neurotrophic factors in a pharmaceutically acceptable carrier.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

EXAMPLE I

MATERIALS AND METHODS

Proteins and Antibodies

Ng-CAM and N-CAM were purified from 14-d embryonic chicken brains by immunoaffinity chromatography using mAbs 10F6 and 3G2 that specifically recognize Ng-CAM, and mAb anti-N-CAM No. 1, respectively. NILE/L1 was purified from 7-day postnatal rat brain using a combination of two other anti-Ng-CAM mAbs, 2C2 and 19H3, that recognize the cytoplasmic region of Ng-CAM, which is highly conserved between chicken Ng-CAM, mouse L1, and rat NILE. The protein purified from detergent extracts of rat brain membranes contains on SDS/PAGE two major components at M_r of 200 kDa and 140 kDa and small amounts of a component at 80 kDa. Polyclonal antibodies against human L1 (kindly provided by Dr. John Hemperly) recognized the 200 kDa and 140 kDa species on immunoblots, confirming that it is NILE/L1.

The 5B8 mAb (obtained from the Developmental Studies Hybridoma Bank) was used to purify N-CAM from 7-day postnatal rat brain; this antibody recognizes cytoplasmic regions of N-CAM. When the rat N-CAM was resolved on SDS/PAGE and stained with Coomassie Blue, the characteristic heterodisperse pattern of polysialylated N-CAM was observed.

Neurocan was isolated and analyzed as described previously (Rauch *et al.*, *supra*; Grumet *et al.*, 1993, *supra*). Brains of 7-day or 2- to 3-month-old Sprague-Dawley rats were extracted with PBS, and proteoglycans were purified by ion exchange chromatography and gel filtration (Kiang et al., 1981). Neurocan was purified by immunoaffinity chromatography using the 1D1 mAb (Rauch

et al., supra). Rat chondrosarcoma chondroitin sulfate proteoglycan (aggrecan) was isolated by CsCl density gradient centrifugation. For studies of the core proteins, proteoglycans were digested for 45-60 min at 37°C with protease-free chondroitinase ABC (Seikagaku America Inc., Rockville, MD) in 100 mM Tris-HCl buffer (pH 8.0 at 37°C) containing 30 mM sodium acetate, and completeness of digestion was confirmed by SDS-PAGE.

Myelin associated glycoprotein (a recombinant form including the ectodomain (Pedraza, L. *et al.*, 1990, *J. Cell Biol. 111*:2651-2661) and epidermal growth factor receptor were kind gifts from Drs. J.L Salzer and J. Schlessinger, respectively.

Commercial reagents included laminin, type I and IV collagens (Collaborative Research), fibronectin (New York Blood Bank, NY), and bovine serum albumin (BSA) (ICN Biomedical, Lisle, IL). Sturgeon notochord chondroitin sulfate, consisting of 80% chondroitin 4-sulfate and 20% chondroitin 6-sulfate, was obtained from Seikagaku America Inc.

MAbs against chicken Ng-CAM were prepared as previously described (Grumet M *et al.*, 1984, *J. Cell Biol. 98*:1746-1756). A mAb specific for the 1D1 proteoglycan has been described previously (Rauch *et al.*, *supra*). The Ig was precipitated from ascites fluid with ammonium sulfate and further purified on DE-52 columns.

Radioligand Binding Assay

Proteoglycans were labeled to a specific activity of 2.5-6 x 10^{18} cpm/mole with ^{125}I by the lactoperoxidase/glucose oxidase method using Enzymobeads (Biorad). Typically, 50 μg in of protein were labeled per reaction. Free iodine was removed by gel filtration with a PD-10 column (Pharmacia, Piscataway, NJ). Binding

assays were performed essentially as described by Zisch, A.H. *et al.*, 1992, *J. Cell Biol. 119*:203-213). One to 30 μg of soluble proteins in binding buffer (16 mM Tris, pH 7.2; 50 mM NaCl; 2 mM $CaCl_2$; 2 mM $MgCl_2$; 0.02 NaN_3) were adsorbed to removable Immulon-2 wells (Dynatech, Chantilly, VA) by overnight incubation at room temperature. Unbound proteins were removed with three washes in binding buffer containing 0.02% Tween- 20, and the wells were blocked by incubation with heat treated BSA/PBB, 1 mg/ml. Wells were then emptied and 50 μl/well of labeled proteins or mixtures of labeled and unlabeled proteins in PBB, 1 mg/ml, were incubated for 2 h at room temperature. Unbound proteoglycan was removed by four washes with TBS (50 mM Tris pH 7.2; 150 mM NaCl; 0.02% Tween-20). Radioactivity bound to wells was determined with a gamma counter. Scatchard plots were generated and the K_d was determined using the MacIntosh version of the Ligand program (Munson *et al.*, 1980, *Anal. Biochem. 107*:220-239).

Cells

Dissociated cells were prepared essentially as described before (Brackenbury, R. *et al.*, *Proc. Natl. Acad. Sci. USA 78*:387-391). In brief, 9-d chick embryo brains were treated with trypsin/EDTA (GIBCO, Grand Island, NY) followed by trituration in DME (GIBCO) containing 10 % fetal calf serum and 50 μg/ml DNase I (Worthington, Freehold, NJ). The cells were washed twice with ITS^+ (Collaborative Research, Bedford, MA)/DME and once by centrifugation through a 3.5% BSA/PBS step gradient.

Substrates

Substrates for cell adhesion and neurite growth assays consisted of a circular array (~1 cm diameter) of

8 to 12 small circular regions that were coated with adsorbed proteins. Coated regions were prepared by incubating 1 to 3.5 μl droplets of protein solutions in a humidified chamber for 30 min (Friedlander *et al.*, 1988, *supra*). After removing the droplets by suction, the dishes were washed 3 times with PBS and blocked with 1% BSA. Coating solutions included both single proteins and mixtures of proteins. For double coats, blocking solution was applied only once, following the second coating. In selected experiments, one of the protein solutions included 1-5 μg/ml rhodamine-labeled BSA as a marker to identify coated regions, which helped in determining the location of boundaries between different substrata.

For quantitative determination of protein binding to plastic, radiolabeled proteins were used to coat dishes following the same procedures used for the cellular assays. After the final wash, the dishes were dried, their walls removed with pliers, and the bottom of the dishes were exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA) to determine the relative amounts of radioactivity in the central region of each spot by using interactive software (ImageQuant, Molecular Dynamics). Absolute values of bound protein were obtained by comparing the relative values with the total radioactivity adsorbed to a similar set of spots that were dried completely without prior washing. For measuring total radioactivity, this method yielded results similar to those obtained with a gamma counter. For measuring surface density of adsorbed proteins, however, the PhosphorImager method is more reliable because it avoids uncertainties that may be caused by differences in spot size and boundary effects.

Cell adhesion assays

250 μl of DME/ITS+ containing 2 to 6 x 10^5 cells were deposited in the central region of 35 mm polystyrene dishes that had been coated with proteins. Following incubation for 80 min at 37°C, unattached cells were removed by washing with PBS and the remaining cells were fixed with 3.5% formalin. Attached cells were counted under a microscope at 200X magnification.

Neurite growth

10^5 brain cells were incubated for 2 days under the same conditions used for cell adhesion assays and were fixed with formalin. Neurite length was defined as the distance between the furthest removed neurite tip and the cell body. Quantitation was done under phase contrast microscopy with the help of a measuring eyepiece.

Immunocytochemistry

The immunocytochemical localization of neurocan, NILE/L1, and N-CAM was performed on sagittal Vibratome sections of 7-d rat cerebellum using the 1D1, 2C2, and 5B8 mAbs, respectively. Rats were perfusion-fixed with picric acid-paraformaldehyde-glutaraldehyde, and sections were stained with peroxidase-conjugated second antibody as described previously (Rauch *et al.*, *supra*).

Analytical Methods

Proteins were resolved on SDS/PAGE and were either transferred to nitrocellulose and immunoblotted with antibodies (Grumet *et al.*, 1984, *supra*; Towbin *et al.*, 1979, *Proc. Natl. Acad. Sci. USA* 76:4350-4354) or stained with Coomassie Blue. Radiolabeled proteins were detected by autoradiography, and protein concentrations were determined using either the Lowry (for neurocan) or the Bradford protein assays (Bio-Rad Laboratories, Richmond,

CA); concentration of aggrecan was determined gravitometrically.

EXAMPLE II

BINDING OF NEUROCAN TO Ng-CAM AND N-CAM

Binding of neurocan to various cell surface proteins including neural CAMs and ECM proteins was determined using a radioligand binding assay (Figure 1). Both rat neurocan and neurocan-C bound to chicken Ng-CAM and to its presumed rat homologue NILE/L1 (Grumet, 1992, *supra*; Sonderegger and Rathjen, 1992, *J. Cell Biol. 119*:1387-1394). Neurocan also bound to N-CAMs from chicken and rat. These results suggest that the proteoglycan binding domains in Ng-CAM and N-CAM have been conserved during the evolution of avian and mammalian species. Neurocan bound to a lesser extent to collagen I but not to myelin-associated glycoprotein (MAG), collagen IV, or EGF-receptor.

Under our standard assay conditions (wells coated with proteins at a concentration of 1.25 μg/ml) neurocan did not bind to laminin but some binding (9% of total counts) was detected when a 10 μg/ml of laminin was used. The fraction of neurocan that bound specifically to Ng-CAM was consistently ~20-25%, with a signal to background ratio as high as 65:1. The percent of neurocan bound to N-CAM was usually lower and varied to a greater extent in different experiments ranging from ~8-15% bound with a signal to background ratio of 18-20:1. By comparison, ^{125}I-labeled aggrecan, a chondroitin sulfate proteoglycan from chondrosarcoma, bound very weakly to Ng-CAM and N-CAM (Figure 1), suggesting that the interactions between neurocan and these neural CAMs are related to structural domains in neurocan.

The radioligand binding assays described above were performed in hypotonic buffer containing 50mM NaCl, which yielded higher signal to background ratios a buffer with 150 mM NaCl. At physiological salt concentration, binding of neurocan to Ng-CAM and N-CAM was diminished by 35-60%, and binding to collagen I was reduced to baseline levels (Figure 2). These results suggest that, *in vivo*, neurocan would not bind to collagen I but it could bind to the neural CAMs. Because only limited amounts of proteoglycans were isolated from rat brain, it was decided to perform saturation and inhibition experiments (see below) using the hypotonic conditions.

To analyze further the binding of neurocan to Ng-CAM and N-CAM, saturation experiments were performed with increasing amounts of labeled proteoglycans. The binding of 7-d neurocan and neurocan-C to both Ng-CAM (Figure 3) and N-CAM (Figure 4) was saturable, indicating the presence of a limited number of neurocan binding sites on Ng-CAM and N-CAM. Scatchard analysis of these data yielded linear plots for the binding of both forms of neurocan to Ng-CAM and N-CAM, indicating a single class of binding sites for 7-d neurocan and neurocan-C. In all four cases, the dissociation constants derived from the plots were quite similar (ranging from 0.21 to 0.42 nM) indicating high affinity binding that is comparable to specific receptor binding.

The specificity of binding of neurocan to these neural CAMs was also investigated in competition experiments (Figure 5). Substrate-bound CAMs and labeled ligands were first incubated with increasing concentrations of unlabeled competitors including neurocan, aggrecan, chondroitin sulfate, chondroitin sulfate disaccharides (resulting from chondroitinase

treatment of the proteoglycans) and fibronectin, and then binding of ^{125}I-neurocan was measured. In these competition experiments, binding of ^{125}I-neurocan-C to Ng-CAM was inhibited by soluble neurocan-C in a concentration-dependent manner. When the labeled neurocan was added at a concentration of 0.1 μg/ml (0.7 nm), 50% inhibition of binding was obtained with unlabeled neurocan-C at a concentration between 0.1 and 0.2 μg/ml (0.7 - 1.4 nM). Half-maximal inhibition was therefore obtained with a 2:1-1:1 molar ratio of unlabeled to labeled ligand, in general agreement with the K_d obtained from the Scatchard analysis.

Significant concentration-dependent inhibition of neurocan binding to Ng-CAM and N-CAM was also produced by free chondroitin sulfate chains (Figure 5). In contrast, sulfated disaccharides were not effective, implying that intact chondroitin sulfate chains are required to inhibit proteoglycan binding to CAMs. In the same assay, fibronectin was ineffective in competing for binding of neurocan. Using the same set of soluble proteins as competitors, similar patterns of inhibition were observed for binding of neurocan-C to N-CAM and for binding of 7-d neurocan to Ng-CAM and to N-CAM. The results suggest that chondroitin sulfate, which constitutes ~20% by weight of neurocan, is important in the binding of neurocan to the neural CAMs. By comparison, equivalent amounts of chondroitin sulfate in the form of free chains were less effective competitors of binding, suggesting that structural features in neurocan in addition to chondroitin sulfate itself are involved in the binding of neurocan to neural CAMs. The combined results suggest that chondroitin sulfate chains may bind weakly to Ng-CAM and N-CAM, and may increase the binding

affinity for CAMs when present on neurocan, but are not sufficient by themselves to support high affinity binding.

To analyze more directly the role of chondroitin sulfate chains in interactions of neurocan with neural CAMs, the binding of native and chondroitinase-treated neurocan to Ng-CAM and N-CAM were compared (Figure 6). Chondroitinase treatment reduced binding of 7-d neurocan and neurocan-C to Ng-CAM by ~70%, and to N-CAM by ~80%. Heat treated proteoglycans retained the ability to bind to the CAMs at levels that were somewhat lower than those of controls. However, chondroitinase treatment followed by heat treatment further reduced the levels of binding nearly to background. These results are consistent with idea that chondroitin sulfate chains on neurocan are involved in binding to Ng-CAM and N-CAM. Nevertheless, the data also indicate that the core protein of neurocan retains specific binding for Ng-CAM and N-CAM even in the absence of chondroitin sulfate chains which allow for higher levels of binding.

EXAMPLE III

COLOCALIZATION OF NEUROCAN, Ng-CAM, AND N-CAM IN DEVELOPING BRAIN

The biological significance of interactions between neurocan and neuronal CAMs would be supported by finding these molecules appearing in at least some of the same locations at certain times during development. The ability to isolate by immunoaffinity chromatography significant amounts of neurocan (using mAb 1D1) as well as NILE/L1 (using anti-Ng-CAM mAbs 2C2 and 19H3) and N-CAM (using mAb 5B8) from 7-d postnatal rat brain is evidence that all these proteins are present at this

stage of development (see Materials and Methods). To determine in more detail the histological localization of these proteins, specific mAbs were used for immunoperoxidase staining of sections of early postnatal rat cerebellum. The staining with mAbs against these three molecules was similar insofar as it was strongest in the molecular layer and in the deeper layers of the cerebellum including the fiber tracts (Figure 7). This general staining pattern was also observed in 4-d and 10-d postnatal brain, and is characteristic of several neural cell adhesion molecules, but differs considerably from the patterns obtained using mAbs against unrelated proteins such as glial fibrillary acidic protein or calbindin.

A more detailed analysis of the staining patterns with the antibodies against neurocan, Ng-CAM and N-CAM revealed subtle differences between them. No staining was detected in the external granule cell layer except for a low level of reactivity with anti-N-CAM. Whereas all three antibodies stained long processes in the fiber tracts, antibodies against neurocan also stained strongly in a region surrounding the fiber tracts. In addition, antibodies against neurocan stained weakly in the Purkinje cell layer (Figure 7, panel b).

Staining experiments in developing rat spinal cord and brain also indicated that Ng-CAM and the proteoglycans nuerocan and 3F8 proteoglycan are co-expressed during development

EXAMPLE IV

EFFECTS OF NEUROCAN ON NEURONAL ADHESION TO Ng-CAM

Given the results described above indicating that neurocan binds with high affinity to Ng-CAM and to N-CAM,

and that these molecules are present in partially overlapping patterns during development, studies were performed examining how neurocan inhibits neuronal adhesion to Ng-CAM in culture. Previous studies from the present inventor's laboratory showed that neurons do not bind to substrates prepared using mixtures containing equal amounts of neurocan and Ng-CAM (Grumet et al.,1993a). In those studies, the two proteins were mixed in solution and then adsorbed simultaneously to the substrates. In the present studies, two different proteins were sequentially adsorbed, and the substrate was then tested for binding of neurons. To help interpret the results, the amounts of protein adsorbed to the substrate for the various combinations of protein concentrations and coating orders were determined (see Materials and Methods, above). The results are shown in Table I, below.

When individual proteins were adsorbed to the substrates, neurons bound strongly to Ng-CAM, weakly to N-CAM, negligibly to BSA, and not at all to neurocan; because of the weak levels of neuronal binding to N-CAM, it was omitted from the double coating experiments described below. As expected, neurons bound to substrates that were coated first (protein 1) with Ng-CAM and then (protein 2) with BSA (Table I). Neurons also bound to substrates that were first coated with BSA and then with Ng-CAM. Even when relatively high concentrations of BSA were adsorbed first, a second adsorption with Ng-CAM was able to support binding of neurons. When substrates were coated first with Ng-CAM and then with high concentrations of neurocan-C, neuronal binding was inhibited (Table I). The degree of inhibition was not closely correlated with the density of

neurocan that bound to the substrate which did not vary significantly for coating solutions in the 11 - 100 μg/ml range. Rather, it was more closely related to the concentration of soluble neurocan that was used to produce the second coat.

These observations suggest that the amount of substrate-bound neurocan *per se* is not the critical factor in causing inhibition. In support of this interpretation, changing the order of the adsorptions, so that the Ng-CAM was added after neurocan, allowed for high levels of neuronal binding (Table I). The promotion of neuronal adhesion by Ng-CAM was observed at all concentrations tested. Qualitatively similar results were obtained for both 7-d neurocan and neurocan-C, however, 7-d neurocan was a slightly more potent inhibitor.

Because neurocan could produce these effects not only by blocking sites on substrate-bound Ng-CAM but also by interacting directly with cell surface ligands including Ng-CAM, it was important to determine whether neurocan had direct effects on cells. To explore this possibility, experiments were done to test the effect of neurocan on the binding of neurons to substrates coated with mAbs against Ng-CAM, which promoted neuronal adhesion (Table II) but did not bind to neurocan. Substrates first coated with BSA and then with anti-Ng-CAM Ig were able to support neuronal attachment, and the degree of attachment increased with the density of the adsorbed antibody. When substrates were coated first with anti-Ng-CAM Ig and then with neurocan, neuronal adhesion was inhibited. The inhibition was most apparent at lower densities of bound anti-Ng-CAM Ig. In addition, substrates coated first with neurocan and then with anti-

Ng-CAM Ig did not support neuronal adhesion even though Ng-CAM was present at densities that supported significant levels of neuronal adhesion. For example, neurons bound to Ng-CAM (0.25 ng/ml) when it was coated following BSA, but no binding was detected even when greater amounts of Ng-CAM (0.42 ng/ml) were obtained on a first coat of neurocan (Table II). Because neurocan does not bind to anti-Ng-CAM, these results suggest that the inhibition was mediated by direct interaction of neurocan with the cell surface.

TABLE I

ADHESION OF CELLS TO SUBSTRATES COATED WITH NEUROCAN AND Ng-CAM

Protein 1	Conc 1 µg/ml	Density 1 ng/mm²	Protein 2	Conc 2 µg/ml	Density 2 ng/mm²	Attached cells cells/mm²
Ng-CAM	50	2.69	**BSA**	100	ND	359 ± 6
	50	2.69		33	ND	375 ± 9
	17	0.75		100	ND	287 ± 74
	17	0.75		11	ND	410 ± 52
BSA	100	ND	**Ng-CAM**	50	2.16	268 ± 33
	33	ND		50	2.54	407 ± 11
	100	ND		17	0.62	270 ± 44
	11	ND		17	0.74	439 ± 15
Ng-CAM	50	2.69	**neurocan-C**	100	1.15	28 ± 13
	50	2.69		33	1.18	163 ± 15
	17	0.75		100	1.02	13 ± 17
	17	0.75		11	1.15	476 ± 69
neurocan	100	1.11	**Ng-CAM**	50	1.39	351 ± 35
	33	1.15		50	1.05	455 ± 26
	100	1.11		17	0.31	456 ± 50
	11	0.96		17	0.41	533 ± 98
Ng-CAM	50	2.69	**7d neurocan**	100	1.56	-6 ± 3
	50	2.69		33	1.22	69 ± 2
	17	0.75		100	1.31	-8 ± 2
	17	0.75		11	1.00	126 ± 61
7d neurocan	100	1.31	**Ng-CAM**	50	1.31	285 ± 26
	33	1.20		50	1.26	440 ± 2
	100	1.31		17	0.41	428 ± 61
	11	1.01		17	0.50	520 ± 26

Substrates were incubated sequentially with soluble protein 1 and protein 2 at the indicated concentrations. The resulting surface densities of adsorbed protein (densities 1 and 2) were determined in parallel experiments using the same substrate, proteins, concentrations and liquid volume as in the cell adhesion assays. To determine density 1, duplicate samples of different concentrations of labelled protein 1 (Ng-CAM, neurocan-C and 7-d neurocan) were spotted on the substrate, and the substrate-bound radioactivity was measured as described in Materials and Methods (sets of identical values of density 1 appear in the table, e.g. 2.69 ng/mm² for Ng-CAM in lines 1, 2, etc., because they were based on the same experimental point. Data not included in the table indicated that the amount of bound radioactivity of protein 1 was not lowered by applying a second protein coat with unlabelled protein). To determine density 2, duplicate spots of unlabelled protein 1 (BSA, Ng-CAM, neurocan-C and 7-d neurocan) were prepared first, then labelled protein 2 (Ng-CAM, neurocan-C and 7-d neurocan) was added, and the bound radioactivity was measured. In the parallel cell adhesion assays with unlabelled proteins, dissociated brain cells from 9 d chick embryos were added to substrates, and the numbers of attached cells after an 80 min incubation period were obtained as indicated in Materials and Methods. Numbers of attached cells were obtained by subtracting the number of cells adhering to the BSA-coated background (20 ± 3) from the total numbers. Data represent averages (n = 2) ± mean deviations. ND = not determined.

TABLE II

Adhesion of Cells to Substrates Coated with Neurocan and anti-Ng-CAM

Protein 1	Conc 1 µg/ml	Density 1 ng/mm²	Protein 2	Conc 2 µg/ml	Density 2 ng/mm²	Attached cells cells/mm²
anti-Ng-CAM	30	1.50	BSA	33	ND	522 ± 32
	10	0.73		33	ND	273 ± 53
BSA	33	ND	anti-Ng-CAM	30	0.55	292 ± 73
	33	ND		10	0.25	208 ± 52
anti-Ng-CAM	30	1.50	neurocan	33	0.41	373 ± 0
	10	0.73		33	0.76	-13 ± 0
neurocan	33	1.20	anti-Ng-CAM	30	0.42	-12 ± 0
	33	1.20		10	0.17	-13 ± 0

See Table I for methods. Numbers of attached cells were obtained by subtracting the number of cells adhering to the BSA-coated background (5 ± 2) from the total numbers. Data represent averages (n = 2) ± mean deviations, where shown. ND = not determined.

EXAMPLE V

EFFECTS OF NEUROCAN ON NEURITE OUTGROWTH

A critical aspect of neuronal development is the growth of processes. Studies were therefore done to explore the effects of neurocan on neurite growth in culture using double-coated substrates prepared essentially as described above for the neuronal adhesion experiments. A major difference in these experiments was that the non-adherent cells were not removed by washing after 80 min of incubation and many cells eventually adhered to substrates even in the presence of neurocan.

On substrates coated first with either Ng-CAM or anti-Ng-CAM Ig and then with BSA, neurons extended numerous processes (Figure 7, panels a and c, respectively). When the second coating was performed using neurocan, neurite extension was dramatically diminished on substrates coated with either Ng-CAM or

anti-Ng-CAM Ig (Figure 7, panels b and d). In quantitative experiments, the neurite length histograms for both Ng-CAM and anti-Ng-CAM substrates (Figure 8) showed a significant fraction of neurites longer than 20 μm and gradually fewer neurites of increasing length. In contrast, neurons grown on neurocan plus either Ng-CAM or anti-Ng-CAM had most neurites in the 0 - 20 μm range with very low levels of longer neurites. The average length of neurites growing on Ng-CAM substrates was 19.3 ± 1.8 in the absence of neurocan and 6.7 ± 1.1 in the presence of neurocan. The respective values for anti-Ng-CAM substrates were very similar, 19.4 ± 1.7 μm in the absence and 6.3 ± 0.9 in the presence of neurocan. The combined results indicate that neurocan is a potent inhibitor of neurite growth both on proteins to which it can bind (*e.g.*, Ng-CAM) and to which it does not bind (*e.g.*, anti-Ng-CAM Ig).

DISCUSSION OF EXAMPLES I-V

The major observations of the studies presented above are that

(a) neurocan binds with high affinity to Ng-CAM and N-CAM, two of the most prevalent neural CAMs that play key roles in cell adhesion, neuronal migration, and axonal growth during development;

(b) these three molecules are coexpressed during critical stages of cerebellar histogenesis; and

(c) neurocan inhibits neuronal adhesion and neurite growth.

Binding of Neurocan to Neural CAMs

Of the CAMs and ECM molecules tested for binding of rat neurocan, chicken Ng-CAM exhibited the strongest

binding, followed by rat NILE/L1 which is presumed to be its mammalian homologue (Grumet, 1992, *supra*; Sonderegger *et al.*, *supra*). Neurocan also bound strongly to chicken and rat N-CAM. The K_d of binding to Ng-CAM and N-CAM obtained by Scatchard analysis was of high affinity (~0.3 nM), while collagen I and laminin showed much lower levels of binding. The potential biological importance of these lower affinity interactions may be more relevant in the peripheral nervous system, where these ECM proteins are more abundant than in developing brain.

The observation that the K_d of neurocan-C binding to Ng-CAM and N-CAM is comparable to that obtained using the full-length proteoglycan, suggests that neurocan-C (which represents the C-terminal half of neurocan) contains at least one binding site for these neural CAMs. This region of neurocan contains a number of motifs that have been implicated in binding of other proteins including two EGF-like repeats, a lectin-like domain, and a complement regulatory protein-like sequence (Rauch *et al.*, *J. Biol. Chem. 267*:19536-19547) . Neurocan-C contains a single 32 kDa chondroitin 4-sulfate chain that is linked at serine-944, whereas three additional potential chondroitin sulfate attachment sites (only two of which are utilized) are present in the N-terminal portion of neurocan. The observation that the binding affinities of neurocan for Ng-CAM and N-CAM are quite similar, raises the possibility that there may be a binding site that is shared by these two CAMs which are comprised of multiple Ig and fibronectin type III domains as well as immunologically similar N-linked carbohydrates epitopes (Burgoon *et al.*m *supra*; Grumet, M *et al.*, 1984, *Proc. Natl. Acad. Sci. USA 81*:267-271).

Several of the experiments above (Figures 5 and 6) suggested a role for chondroitin sulfate in the function of neurocan. Little is known regarding the mechanism of inhibition by these chondroitin sulfate containing molecules. However, the present findings suggest that Ng-CAM and N-CAM may be specific neuronal receptors for neurocan. In addition, proteoglycans that have previously been identified in non-neural tissues may also be found in the brain. For example, aggrecan, which was first identified in cartilage, was found by the present inventor and his collaborators to be in rat brain; the primary structure of its core protein has regions that are homologous to those in neurocan (Rauch *et al.*, 1992, *supra*).

Glycosaminoglycans usually occur in tissues only in the form of proteoglycans, although some proteins occur in both glycanated and non-glycanated forms ("part time" proteoglycans), *e.g.*, the amyloid ß precursor protein, chromogranin A, invariant chain, lymphocyte homing receptor (Margolis, R.K. *et al.*, 1993, *Experientia 49*:429-446; Ruoslahti, 1989, *J. Biol. Chem. 264*:13369-13372). While there is no evidence that neurocan occurs in brain without its chondroitin sulfate chains, the present results indicate that such a molecule would bind to Ng-CAM and N-CAM.

Colocalization of Neural CAMs with Neurocan

The immunolocalization experiments demonstrate that neurocan, Ng-CAM/L1/NILE, and N-CAM are prevalent during brain development and colocalize extensively at least in the cerebellum. Inasmuch as the anti-N-CAM mAb used above recognizes the cytoplasmic region of N-CAM, it revealed only the larger N-CAM species that parallels the expression pattern of Ng-CAM/L1/NILE in developing

cerebella (Pollerberg, E.G. *et al.*, 1985, *J. Cell Biol.* *101*:1921-1929). Developing cerebellar neurons express Ng-CAM/L1/NILE and N-CAM *in vitro*, and recent *in situ* hybridization histochemistry studies by the present inventor's collaborators indicate that mRNA for neurocan is synthesized by granule cells in rat cerebella. These results suggest that granule cells may be major contributors to the high levels of neurocan that appear in the molecular layer of the developing cerebellum.

These observations, together with the effects of mixtures of neurocan and Ng-CAM on cells, raise the possibility of at least two opposing but not exclusive modes of action that may occur *in vivo*:

(1) Binding of neurocan to Ng-CAM, and other cell surface proteins including N-CAM, results in inhibition of neuronal adhesion and axonal migration consistent with the hypothesis that proteoglycans act as barriers against neuronal penetration (Perris, R. *et al.*, 1991, *Development* *111*:583-599; Oakley, R.A. *et al.*, 1991, *Dev. Biol.* *147*:187-206; Snow, D.M. *et al.*, 1990, *Exp. Neurol.* *109*:111-130; Pindzola, R.R. *et al.*, 1993, *Dev. Biol.* *156*:34-48; Cole, G.J. *et al.*, 1991, *Neuron* 7:1007-1018. This could account for the observation that the parallel processes of developing granule cells do not enter the molecular layer in the developing cerebellum (Jacobson, 1991, *supra*; Grumet, M. *et al.*, 1993a, *J. Cell Biol.* *120*:815-824).

(2) Binding of Ng-CAM (and possibly other CAMs such as N-CAM) to neurocan results in neutralization of the inhibitory effects of the proteoglycan.

Given that substantial amounts of neurocan can be extracted simply with PBS from developing brain, it is likely that this secreted proteoglycan can diffuse

locally between cells and bind to Ng-CAM and N-CAM. Whereas these CAMs are found primarily associated with the plasma membrane, small proportions of extracellular forms and large proteolytic fragments of these CAMs have been found in extracts prepared from brain tissue. For example, chicken Ng-CAM as well as mammalian L1/NILE have large forms that may be released from neurons in response to stimuli or as a result of proteolytic cleavage (Burgoon *et al.*, *supra*; Richter-Landsberg, C. *et al.*, 1984, *J. Neurochem.* *43*:841-848; Sweadner, K.J., 1983, *J. Neurosci.* *3*:2504-2517; Sadoul, K. *et al.*, 1988, *J. Neurochem.* *50*:510-521; Nybroe, O. *et al.*, 1990, *Int. J. Dev. Neurosci* *8*:273-281).

Although the radioligand assay revealed high affinity binding of radiolabeled neurocan to Ng-CAM, binding of soluble labeled Ng-CAM to immobilized neurocan was not found, despite the fact that labeled Ng-CAM binds to substrate bound Ng-CAM (Grumet, M. *et al.*, 1993b, *Cell Adhesion Communic.* *1*:177-190). It is possible that the ability of neurocan to bind to Ng-CAM depends on its configuration which could be modified by binding to a substrate. Attempts to present neurocan in a different conformation, for example, by using monoclonal antibodies against neurocan as a linker to the substrate did not yield significant levels of Ng-CAM binding. In addition, the present inventor observed that when neurocan was bound to Covaspheres, it coaggregated with Ng-CAM coated Covaspheres only when the neurocan had been treated with chondroitinase. It is possible that concentrating the highly charged neurocan either on a substrate or on the surface of Covaspheres produces a highly charged local environment that may inhibit ligand binding. This affect and the fact that Ng-CAM is usually found in a membrane

and binds in a highly cooperative manner, may explain the inability to measure binding of soluble Ng-CAM to substrate bound neurocan. In any case, the solubility of neurocan and its binding properties are consistent with its high level of co-localization with Ng-CAM and N-CAM *in vivo*.

Effects of Neurocan on Neurons

The present results support the notion that neurocan binding to neurons modulates their behavior. Using a centrifugation binding assay the present inventor's group previously found that neurons could bind to substrates coated with neurocan and that the 1D1 antibody specifically inhibited this binding (Grumet et al.,1993a, *supra*). In the present study, experiments with cells demonstrated that neurocan inhibited neuronal adhesion and neurite growth, although the mechanism for this effect was not clear. The "inhibitor" may act enzymatically to alter CAMs and inactivate them. In this regard, no change in the mobility of Ng-CAM on gels has been observed following incubation in physiological buffers with neurocan (Grumet et al.,1993a). A second possibility is that the "inhibitor" binds to the cell surface either to block an adhesion molecule and/or to generate a signal into the cell. This possibility appears more likely inasmuch as Fab' fragments of antibodies against Ng-CAM and N-CAM inhibited the binding of ^{125}I-neurocan to neurons.

The ability of cells to respond negatively to neurocan suggests that its binding to cell surface molecules such as Ng-CAM and N-CAM may generate signals that influence the response of the cell. In this regard, recent studies indicate that binding of other ligands to both of these CAMs at the surface of neurons (Schuch *et*

al., 1989, *Neuron* 3:13-20; von Bohlen und Halbach, F. *et al.*, 1992, *Eur. J. Neurosci.* 4:896-909) and binding of chondroitin sulfate proteoglycans to growth cones (Snow, D.M. *et al.*, 1993, *J. Neurobiol.* 23:322-336) produced increases in intracellular levels of calcium, but the mechanisms of transmembrane signalling mediated by these interactions are unclear. These observations raise the possibility that the inhibitory effects of neurocan, either directly or indirectly, involve its binding to Ng-CAM and N-CAM on the cell surface.

The cellular assays employed here were designed to investigate the mechanisms by which neurocan influences neuronal behavior. When anti-Ng-CAM antibodies were used as a permissive substrate, neurocan inhibited neuronal adhesion and neurite growth. Because neurocan does not bind to anti-Ng-CAM, the results suggest that neurocan inhibited neuronal adhesion and neurite growth by interacting directly with the cell surface.

The interpretation of the effects of neurocan on the adhesion of neurons to Ng-CAM itself is somewhat more complex due to potential interactions of neurocan with both substrate-bound Ng-CAM and the neuronal plasma membrane. When Ng-CAM was used as a permissive substrate, inhibition occurred only when neurocan was incubated on the substrate after Ng-CAM had been adsorbed, and not when neurocan was coated before Ng-CAM. This occurred even when the amounts of neurocan adsorbed to the substrate were the same. Thus, the ability of the mixed substrates to support neuronal adhesion depended not only on the amounts of the proteins, but also on their configuration on the substrate. A likely interpretation, based on the high binding affinity of soluble neurocan to immobilized Ng-CAM, is that at least

part of the inhibition was due to binding of neurocan to substrate-bound Ng-CAM. In support of this view, when higher concentrations of soluble neurocan (which causes neurocan to bind to a larger proportion of the Ng-CAM, Figure 3) were incubated with Ng-CAM-bound substrates, higher levels of inhibition were observed, (Table I).

Analysis of neurite outgrowth also indicated that neurocan is a potent inhibitor of this critical aspect of neuronal development. These results are important because there is not always a direct relationship between the ability of a particular protein to promote neuronal adhesion and neurite growth (Lemmon, V. *et al.*, 1992, *J. Neurosci. 12*:818-826; Calof, A.L. *et al.*, 1991, *J. Cell. Biol. 115*:779-794). The results of the current study suggest that whether a particular region will promote, allow, or inhibit cell adhesion and axonal growth will depend not only on the relative amounts of the CAMs and proteoglycans but also on the their sequences of expression and organization during development.

EXAMPLE VI

TRANSFECTION OF NG-CAM INTO MAMMALIAN CELLS

To analyze the function of Ng-CAM and L1 expressed in mammalian cells, expression vectors have been constructed for the transfection of Ng-CAM into mammalian cells.

One construct includes the cDNA for most of the extracellular region of Ng-CAM that is linked to a signal sequence for phospholipid attachment to the plasma membranes. Expression of this construct in CHO cells indicated that Ng-CAM was localized to the plasma membrane. Experiments are in progress to analyze the

binding properties of this transfected form of Ng-CAM as well as mutated forms of this molecule and the human L1 protein.

The cells transfected to express Ng-CAM bind to Ng-CAM and to chondroitin sulfate proteoglycans from brain including 3F8 proteoglycan and neurocan. These results suggest that this form of the molecule retains at least some of the activities of the native Ng-CAM protein.

Such cells will also be cotransfected with a construct encoding phospholipase D at the cell surface. Such a double transfection should result in a cell line that has the capability of synthesizing a membrane anchored glycoprotein that is constitutively released because of the action of the phospholipase D enzyme at the surface of the same cell. This provides a useful system for analyzing functions of the cell adhesion molecules. In addition, it may provide new technologies for producing and releasing various membrane proteins from cells for therapeutic purposes.

EXAMPLE VII

Implantation of Tubes containing Ng-CAM in a Support Matrix into Rats

To analyze the ability of Ng-CAM to promote regeneration in animals, and as a therapeutic model, Ng-CAM protein will be incorporated into silicone tubes. As described previously (LeBeau *et al.*, *supra*), the proximal and distal stumps of severed rat sciatic nerves are sutured into the openings of silicone tubes. The insides of the tubes are filled with a saline solution containing 1-100 μg/ml of Ng-CAM. Albumin serves as a control protein. At various intervals following the initial surgery, the ability of the Ng-CAM to promote recovery of

neuromuscular function is assessed in the live animals by measuring evoked muscle action potentials in the gastrocnemius muscles, as described by Archibald, *et al.* (*supra*). The nerves are then surgically removed from the rats, fixed and analyzed by microscopy for nerve regrowth.

Based on the ability of Ng-CAM to promote the growth of neurites, it is expected that Ng-CAM will accelerate the rate of the regeneration process and increase the maximal level of regeneration and recovery of neuromuscular function.

EXAMPLE VIII

Implantation of Tissue Guides Impregnated with Ng-CAM in into Rats

As another method to analyze the ability of Ng-CAM to promote regeneration in animals, and as a therapeutic model, Ng-CAM protein is incorporated into collagen based nerve guides as conduits for peripheral nerve regeneration. As described by Archibald *et al.* (*supra*), the proximal and distal stumps of severed rat sciatic nerves are sutured into the collagen based nerve guides. The collagen guides are impregnated with a saline solution containing 1-100 μg/ml of Ng-CAM or a control protein (such as albumin). At various intervals following initial surgery, the ability of the Ng-CAM to promote recovery of neuromuscular function is assessed in the live animals by measuring evoked muscle action potentials in the gastrocnemius muscles, as described by Archibald, *et al.* (*supra*). The nerves are then surgically removed from the rats, fixed and analyzed by microscopy for nerve regrowth (LeBeau *et al.*, *supra*).

Based on the ability of Ng-CAM to promote the growth of neurites, it is expected that Ng-CAM will accelerate the rate of the regeneration processes and increase the maximal level of regeneration and recovery of neuromuscular function.

The references cited above are all incorporated by reference herein, whether specifically incorporated or not.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method of promoting the regeneration of a nerve in a subject having nerve damage, comprising administering to a subject in need of such treatment an amount of Ng-CAM, or a functional derivative thereof, effective in promoting regeneration of nerves.

2. A method according to claim 1 wherein said nerve damage is peripheral nerve damage and said regeneration is of a peripheral nerve.

3. A method according to claim 2 wherein said method further comprises administering to said subject, in combination with said Ng-CAM or functional derivative, an effective amount of at least one other agent that is capable of promoting neuronal survival, growth, differentiation or regeneration.

4. A method according to claim 3 wherein the other agent is selected from the group consisting of nerve growth factor, brain-derived neurotrophic factor, ciliary neurotrophic factor, neurotrophin-3, neurotrophin-4, neurotrophin-5 and laminin.

5. A method according to claim 1 wherein the nerve damage is associated with physical or surgical trauma, infarction, bacterial or viral infection, toxin exposure, gliosis, degenerative disease, malignant disease, Parkinson's disease or Alzheimer's disease.

6. A method according to claim 2 wherein the peripheral nerve damage is associated with physical or

surgical trauma, infarction, bacterial or viral infection, toxin exposure, degenerative disease or malignant disease.

7. A method according to claim 1 wherein said Ng-CAM or functional derivative is administered in a form associated with a solid or semisolid phase support material.

8. A method according to claim 7, wherein said solid or semisolid phase support material is a collagen gel.

9. A pharmaceutical composition useful in the treatment of nerve damage, comprising:

(a) an amount of Ng-CAM or a functional derivative thereof effective for treating nerve damage; and

(b) a pharmaceutically acceptable carrier.

10. A pharmaceutical composition according to claim 9, further comprising

(c) at least one other agent that is capable of promoting neuron survival, growth, differentiation or regeneration.

11. A pharmaceutical composition according to claim 9, wherein said nerve damage is peripheral nerve damage.

12. A pharmaceutical composition according to claim 10, wherein said nerve damage is peripheral nerve damage.

13. A method of promoting regeneration of a injured or severed nerve, comprising exposing an injured or severed nerve to a concentration of Ng-CAM or a functional derivative thereof that is effective in promoting the regeneration of neurons.

14. A method according to claim 13, that is carried out *in vitro*.

15. A method according to claim 13, that is carried out *in vivo*.

16. A method according to claim 13, wherein said injured or severed nerve is surgically entubulated in an entubulation device which contains an amount of Ng-CAM or a functional derivative thereof effective in promoting said regeneration.

17. A method for promoting neuronal survival or neurite growth by neutralizing or overcoming the inhibitory effect of a chondroitin sulfate proteoglycan on said survival or growth, which method comprises contacting a nerve fiber inhibited in its survival or growth by a chondroitin sulfate proteoglycan with an amount of Ng-CAM effective in neutralizing or overcoming said inhibitory effect, thereby promoting neuronal survival or neurite growth.

18. The method of claim 17, wherein said proteoglycan is neurocan.

19. A method of diagnosing a neuronal disorder associated with an abnormal level of a substance which binds to Ng-CAM in a subject, comprising:

(a) measuring the level of said Ng-CAM-binding substance in a sample from said subject; and

(b) comparing the levels of said substance measured in step (a) with the level of said substance in an analogous sample from a normal individual or a standard level of said substance,

thereby detecting an abnormality in the level of said Ng-CAM-binding substance in said subject, said abnormality being diagnostic of the neuronal disorder.

20. A method according to claim 19, wherein said Ng-CAM-binding substance is a chondroitin sulfate proteoglycan.

21. A method according to claim 20, wherein said proteoglycan is the 3F8 proteoglycan.

22. A method for identifying a compound or agent which binds to Ng-CAM or to a functional derivative thereof, comprising:

(a) exposing said compound or agent to Ng-CAM or a functional derivative thereof;

(b) measuring the binding of said compound or agent to said Ng-CAM or functional derivative.

23. A method according to claim 20, wherein said Ng-CAM or to a functional derivative is immobilized on a solid phase support or carrier.

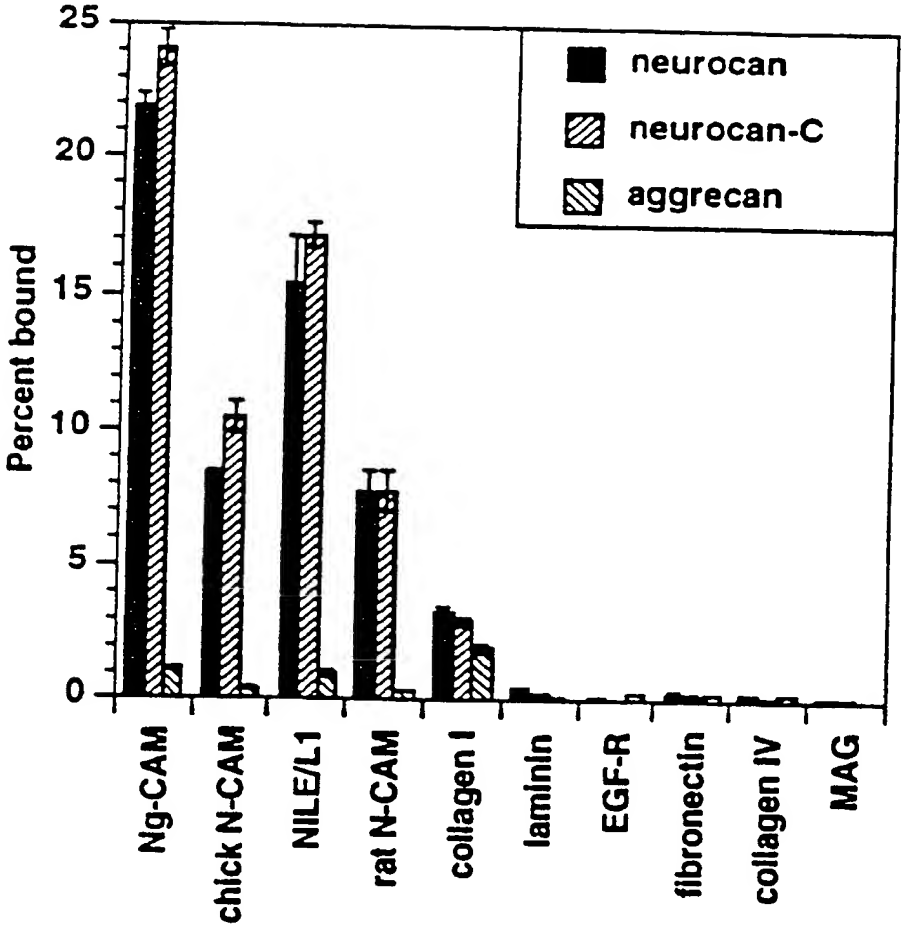

25
20
15
10
5
0
Percent bound
neurocan
neurocan-C
aggrecan
Ng-CAM
chick N-CAM
NILE/L1
rat N-CAM
collagen I
laminin
EGF-R
fibronectin
collagen IV
MAG

FIG. 1

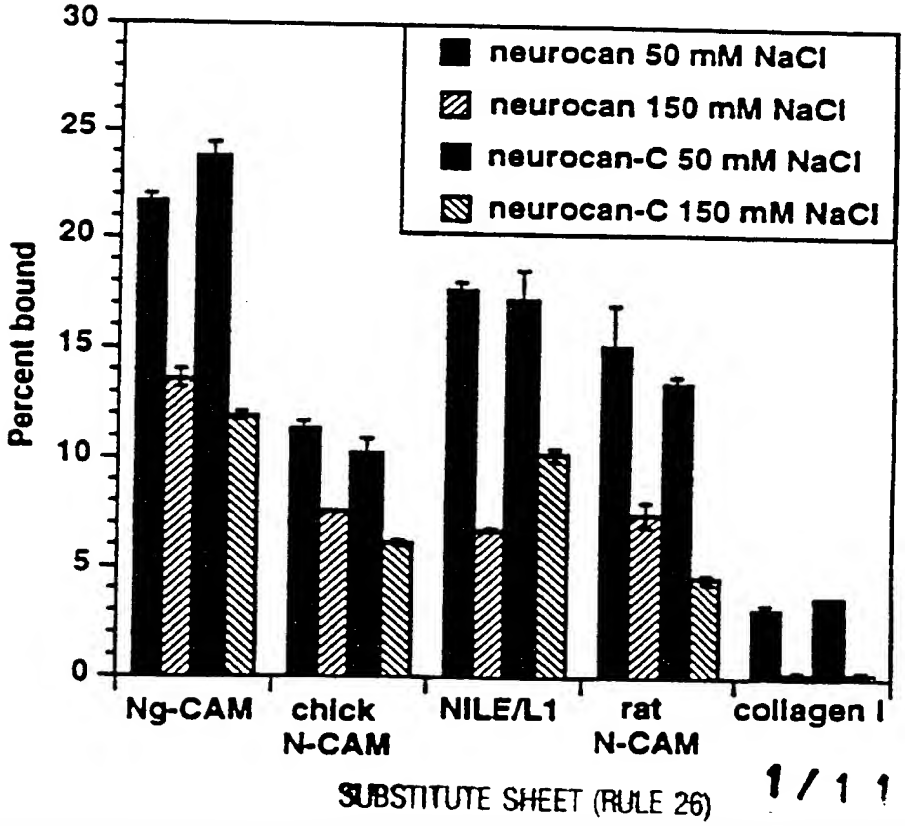

30
25
20
15
10
5
0
Percent bound
neurocan 50 mM NaCl
neurocan 150 mM NaCl
neurocan-C 50 mM NaCl
neurocan-C 150 mM NaCl
Ng-CAM
chick N-CAM
NILE/L1
rat N-CAM
collagen I

FIG. 2

FIG. 3A

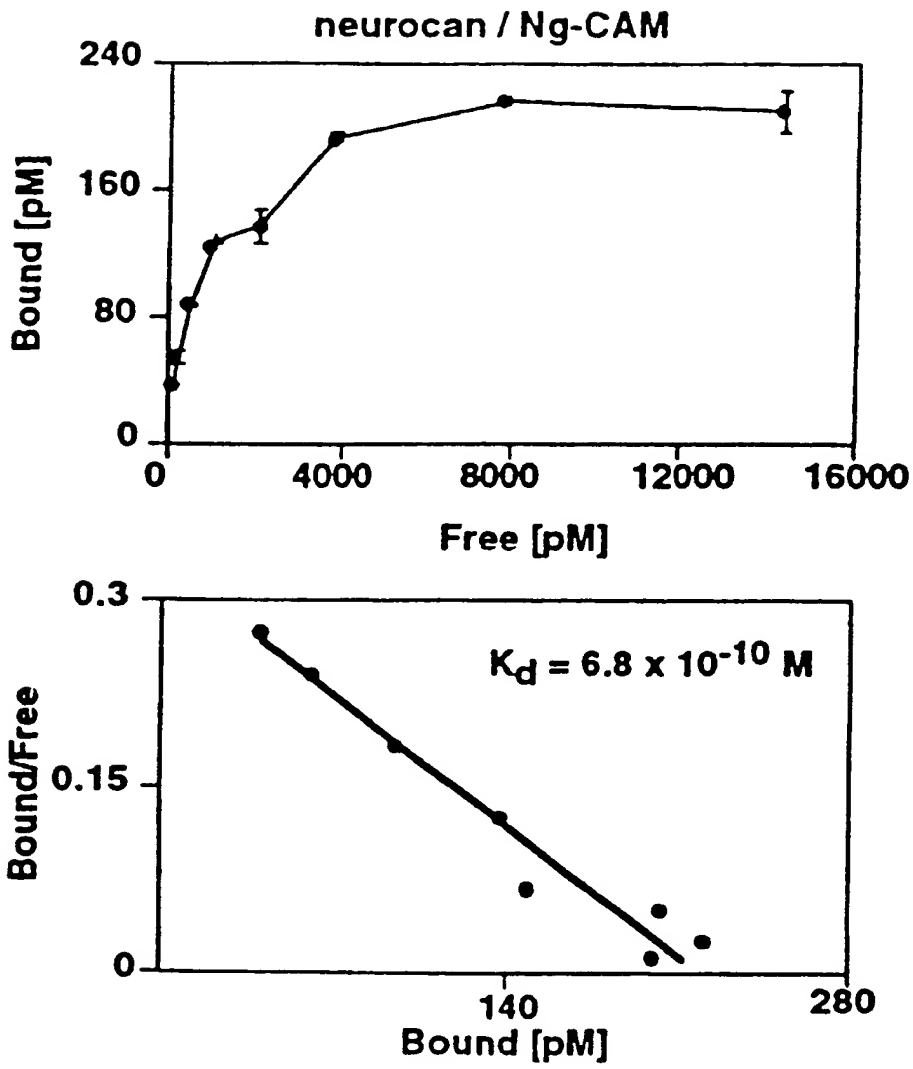

neurocan / Ng-CAM
240
160
80
0
Bound [pM]
0
4000
8000
12000
16000
Free [pM]
0.3
0.15
0
Bound/Free
$K_d = 6.8 \times 10^{-10}$ M
140
280
Bound [pM]

FIG. 3B

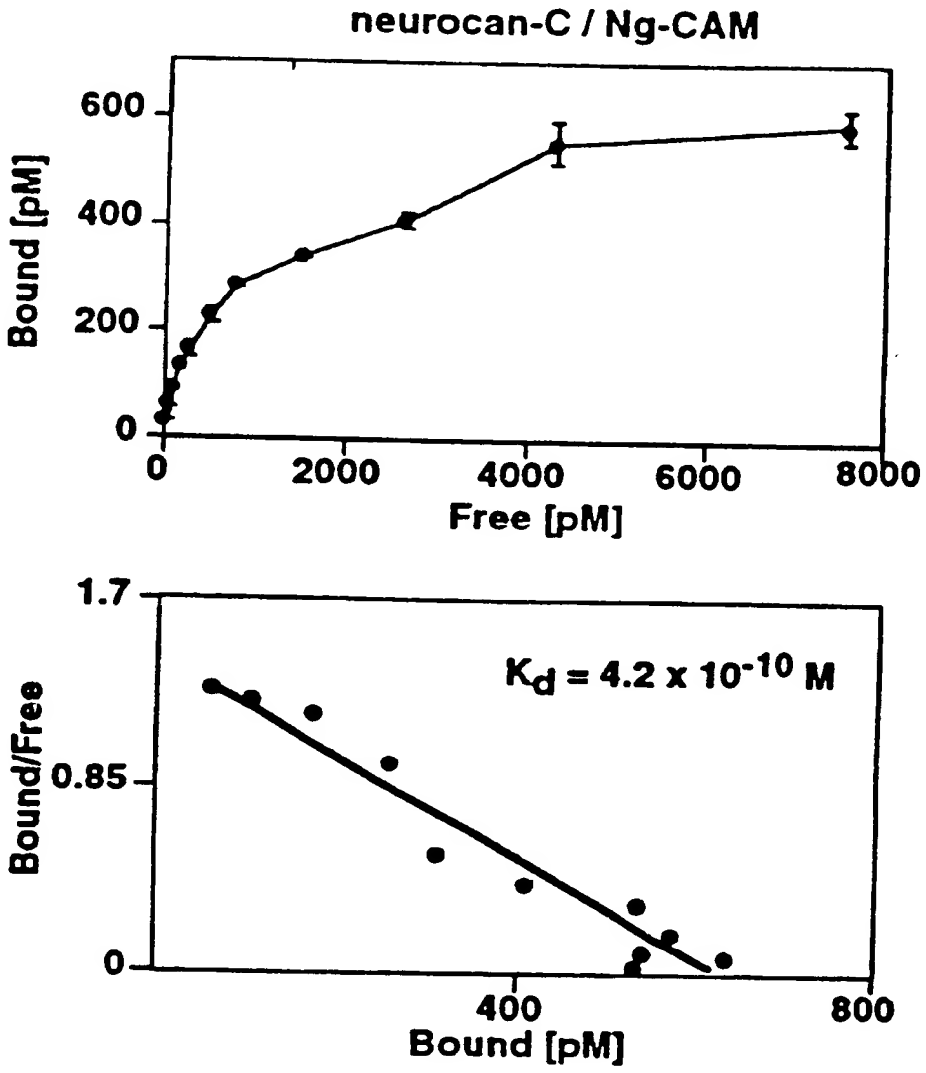

neurocan-C / Ng-CAM
Bound [pM]
600
400
200
0
0
2000
4000
6000
8000
Free [pM]
Bound/Free
1.7
0.85
0
$K_d = 4.2 \times 10^{-10}$ M
400
800
Bound [pM]

FIG. 4A

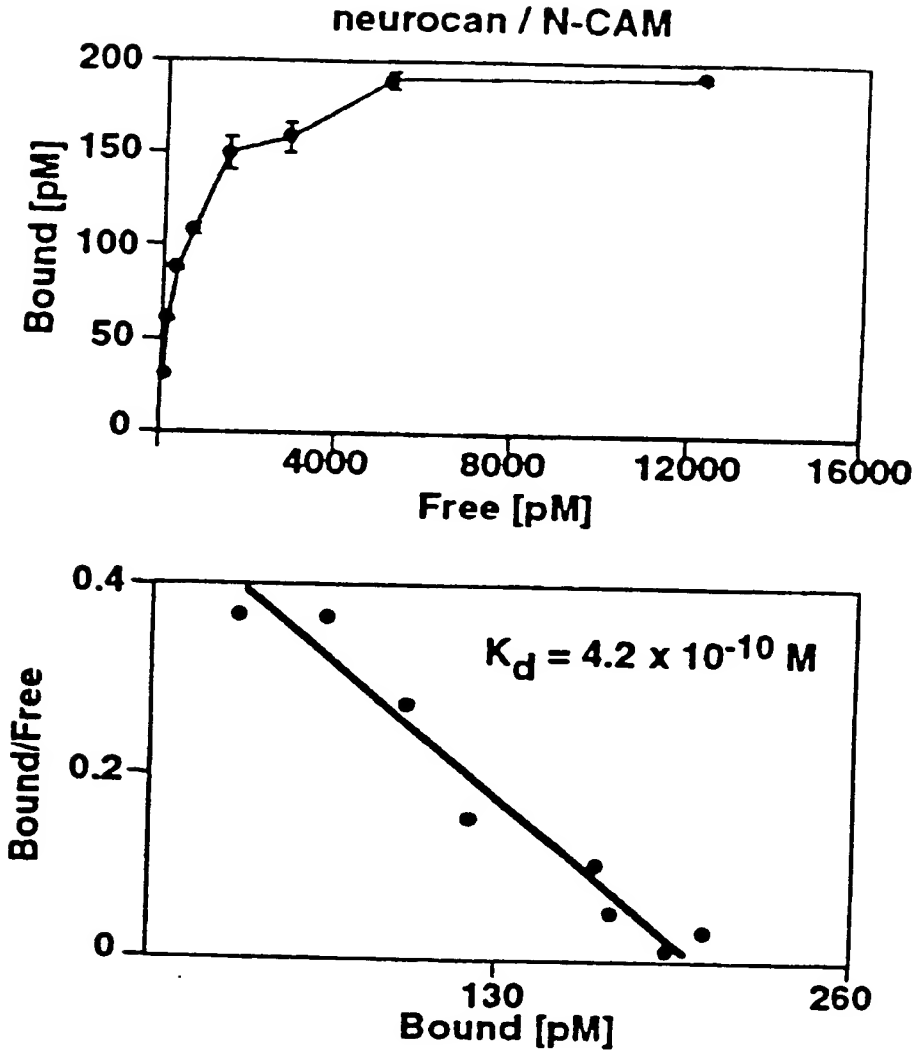
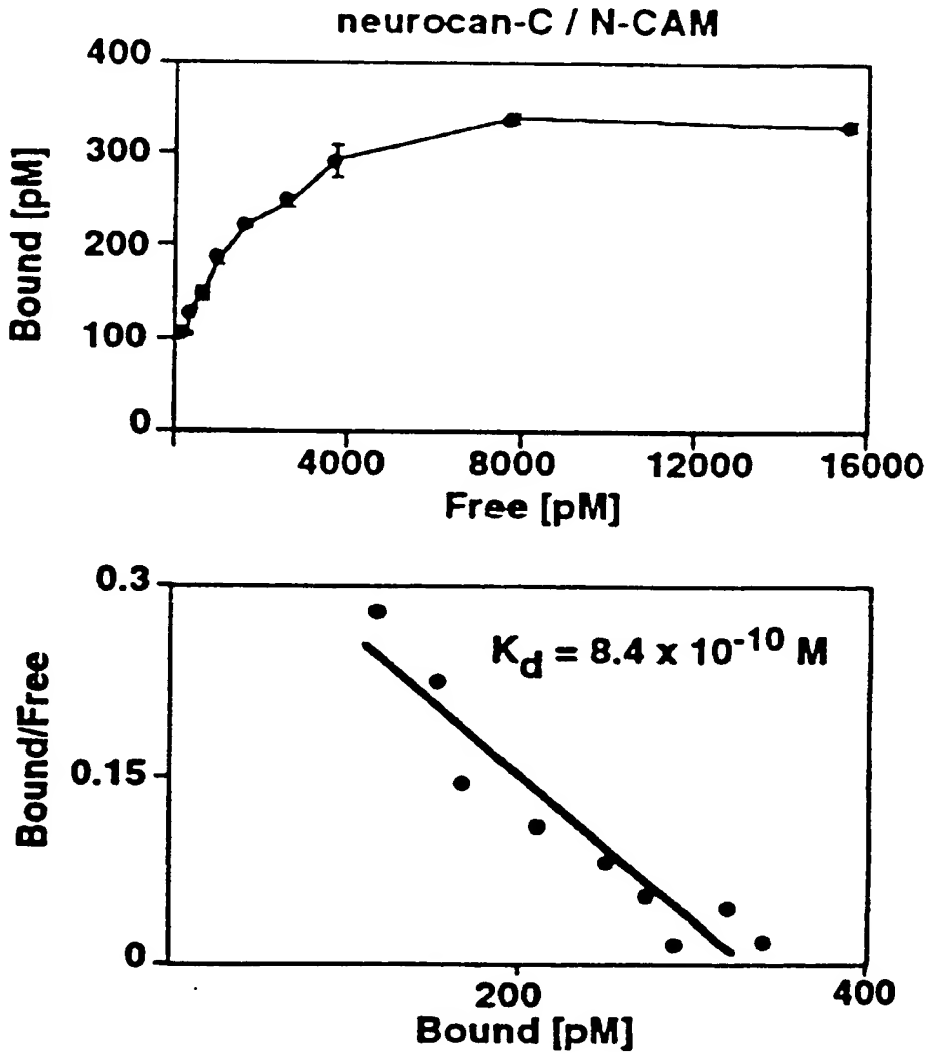

neurocan / N-CAM
200
150
100
50
0
Bound [pM]
4000
8000
12000
16000
Free [pM]
0.4
0.2
0
Bound/Free
$K_d = 4.2 \times 10^{-10}$ M
130
260
Bound [pM]

FIG. 4B


neurocan-C / N-CAM
Bound [pM]
400
300
200
100
0
4000
8000
12000
16000
Free [pM]
Bound/Free
0.3
0.15
0
$K_d = 8.4 \times 10^{-10}$ M
200
400
Bound [pM]

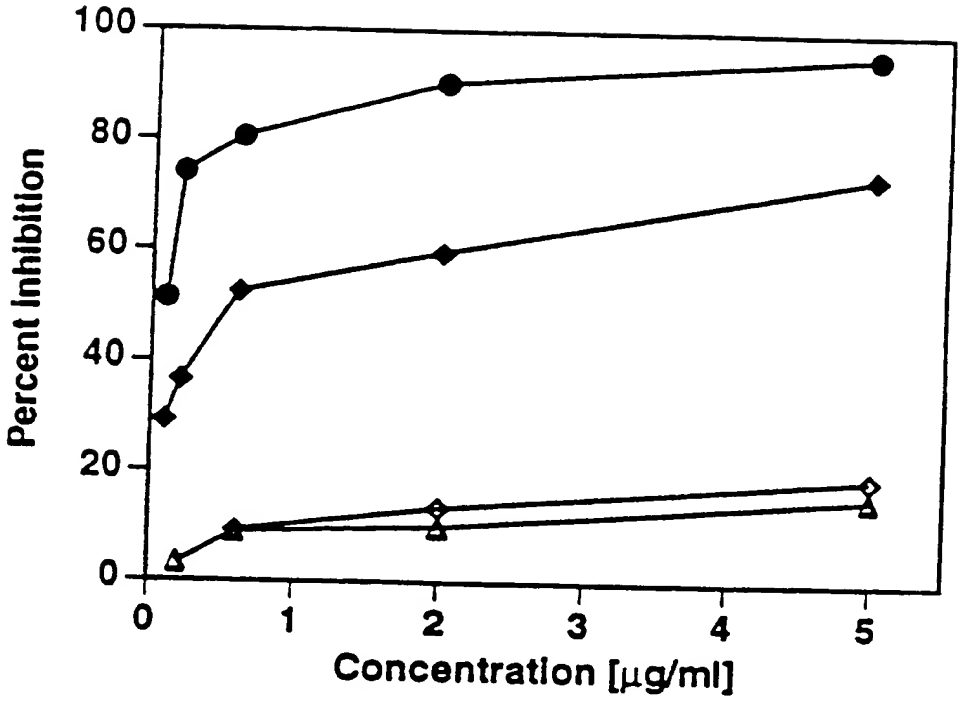

100
80
60
40
20
0
Percent Inhibition
0
1
2
3
4
5
Concentration [µg/ml]

FIG. 5

FIG. 6A

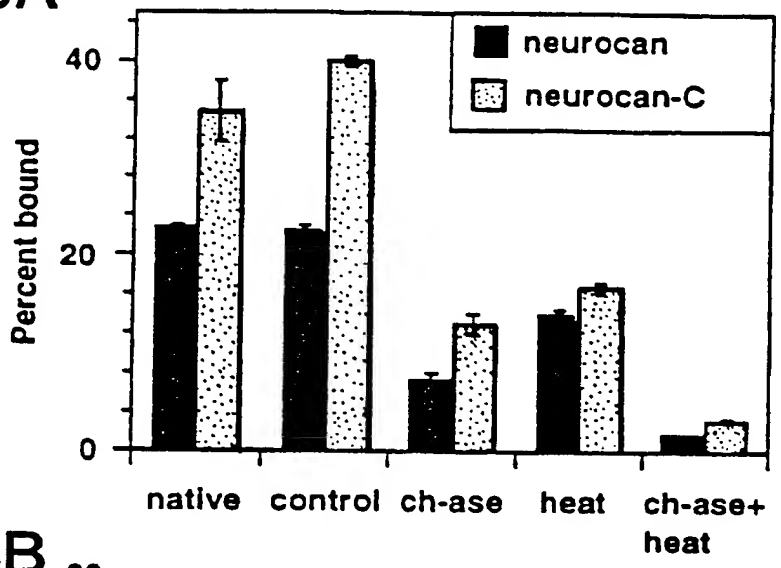

neurocan
neurocan-C
Percent bound
40
20
0
native
control
ch-ase
heat
ch-ase+ heat

FIG. 6B

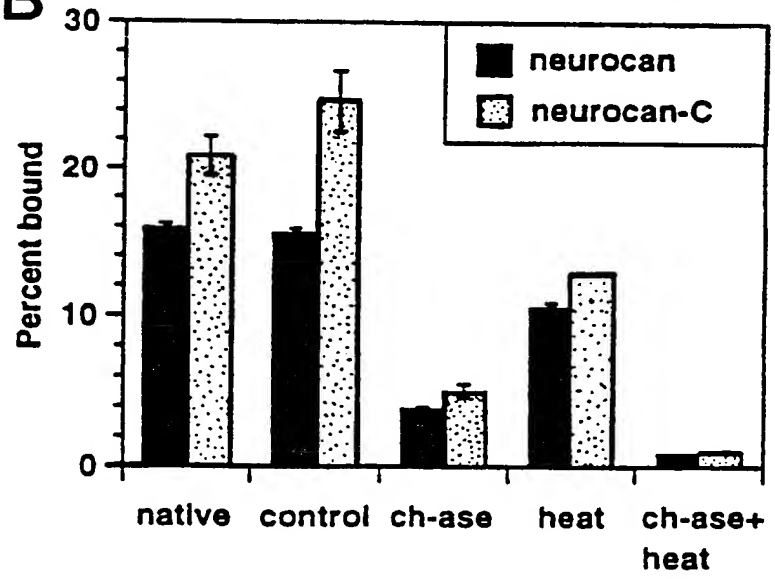

neurocan
neurocan-C
Percent bound
30
20
10
0
native
control
ch-ase
heat
ch-ase+ heat

FIG. 7C

FIG. 7B

FIG. 7A

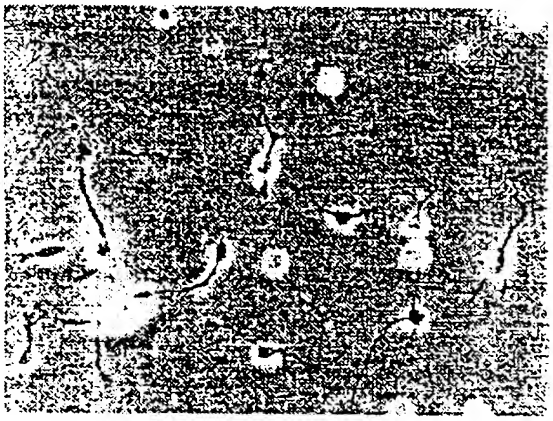


FIG. 8A

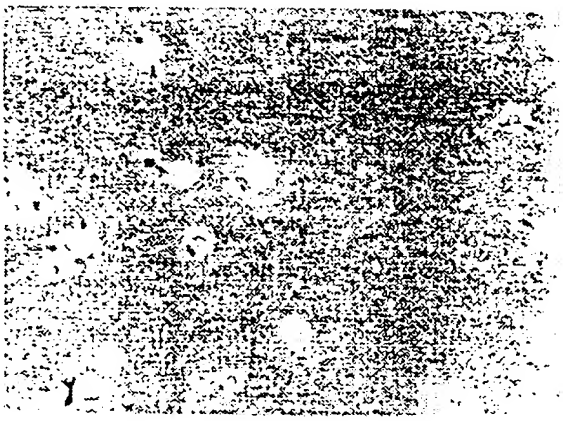


FIG. 8B

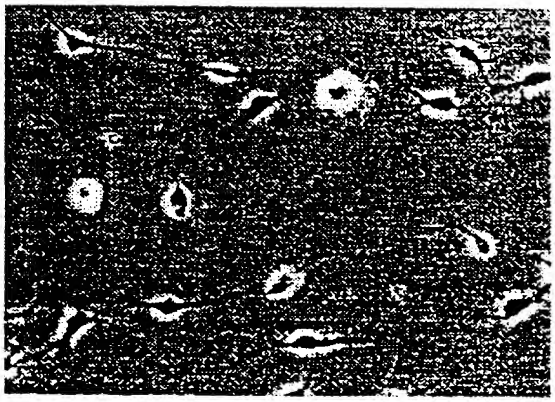


FIG. 8C

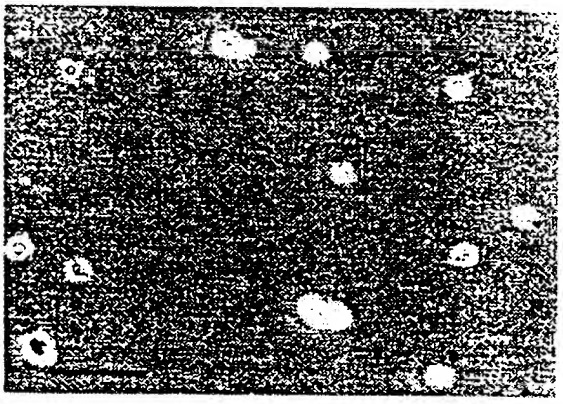


FIG. 8D

FIG. 9A

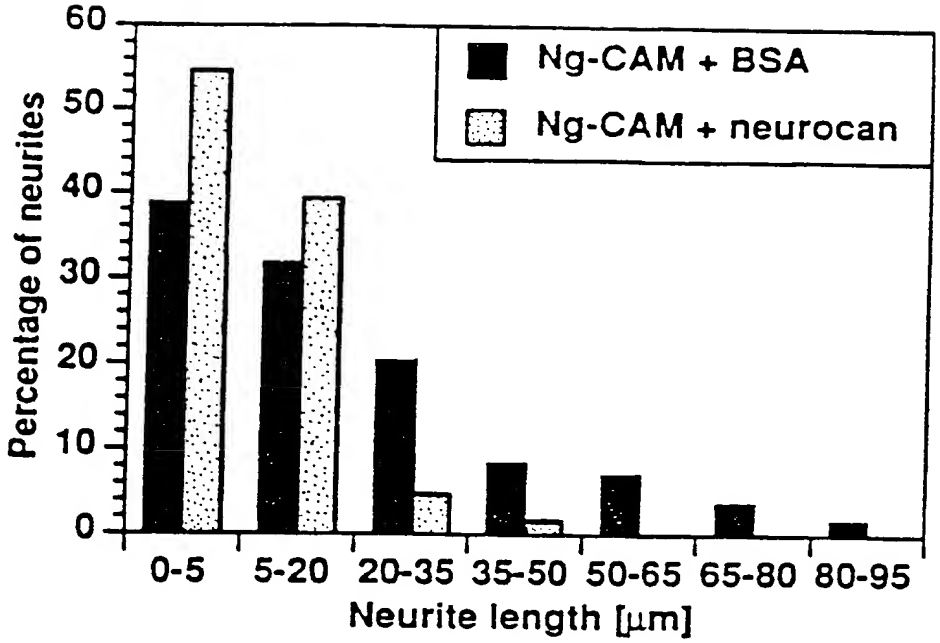
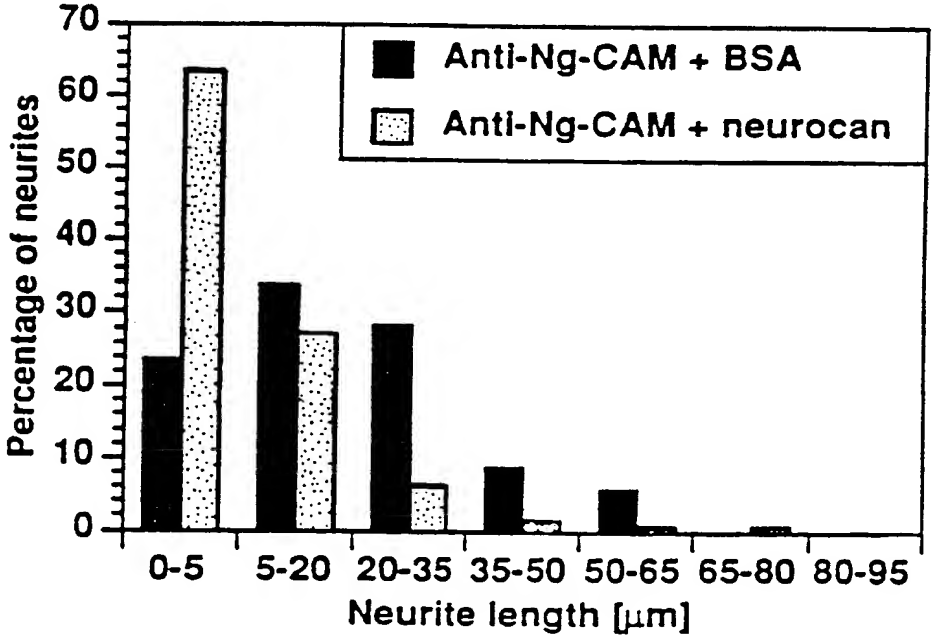

Ng-CAM + BSA
Ng-CAM + neurocan
Percentage of neurites
0
10
20
30
40
50
60
0-5
5-20
20-35
35-50
50-65
65-80
80-95
Neurite length [μm]

FIG. 9B


Anti-Ng-CAM + BSA
Anti-Ng-CAM + neurocan
Percentage of neurites
0
10
20
30
40
50
60
70
0-5
5-20
20-35
35-50
50-65
65-80
80-95
Neurite length [μm]

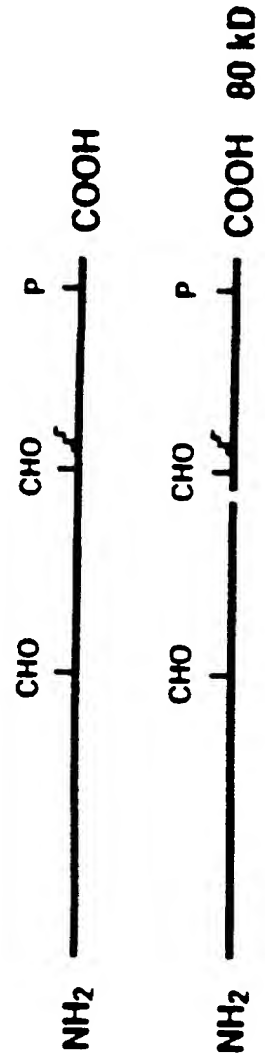


FIG. 10A

FIG. 10B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12858

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 14/435, 14/46; A61K 38/16, 49/00; C12N 1/38; G01N 33/50
US CL : 514/2; 530/350; 435/244; 435/7.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2; 530/350; 435/244; 435/7.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS, Medline, BIOSIS, WPIDS
search terms: ng cam, neuron glia cell adhesion molecule, nerve regeneration, repair, proteoglycan, disease, disorder

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	The Journal of Cell Biology, Volume 107, issued July 1988, J.L. Bixby et al., "Identification of the major proteins that promote neuronal process outgrowth on Schwann cells in vitro", pages 353-361, see entire document.	9,11,13,14 1-8,10,12, 15-23
Y	US, A, 4,955,892 (DANILOFF) 11 September 1990, see entire document.	1-8, 15-18
Y	The Journal of Cell Biology, Volume 102, Number 102, issued February 1986, D.R. Friedlander et al., "Nerve growth factor enhances expression of neuron-glia cell adhesion molecule in PC12 cells", pages 413-419, see entire document.	3,4,10,12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 06 FEBRUARY 1995	Date of mailing of the international search report 17 FEB 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Jacqueline G. Krikorian Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12858

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P Y, P	The Journal of Cell Biology, Volume 125, Number 3, issued May 1994, D.R. Friedlander et al., "The neuronal chondroitin sulfate proteoglycan neurocan binds to the neural cell adhesion molecules Ng-CAM/L1/NILE and N-CAM, and inhibits neuronal adhesion and neurite outgrowth", pages 669-680, see entire document.	9,11,13, 14,22,23 1-8,10,12, 15-21
Y	The Journal of Cell Biology, Volume 120, Number 3, isssued February 1993, M. Grumet et al., "Functional characterization of chondroitin sulfate proteoglycans of brain: interactions with neurons and neural cell adhesion molecules", pages 815-824, see entire document.	17-23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12858

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

I.Group I, claims 1-16, drawn to a method of promoting the regeneration of a nerve in a subject, comprising administering Ng-CAM or a functional derivative thereof, and to a pharmaceutical composition comprising Ng-CAM or a functional derivative thereof.

II.Group II, claims 17 and 18, drawn to a method of promoting neuronal survival or neurite outgrowth, comprising contacting a nerve fiber with Ng-CAM or a functional derivative thereof.

III.Group III, claims 19-23, drawn to a method of diagnosing a neuronal disorder comprising measuring the level of Ng-CAM binding substance, and to method of identifying a compound or agent which binds to Ng-CAM.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features to form a single inventive concept. A method of promoting the regeneration of a nerve in a subject (Group I) is distinct from a method of diagnosing a neuronal disorder (Group III), and is distinct from promoting neuronal survival and neurite outgrowth (Group II). Neurite outgrowth and neuronal survival may occur without promoting nerve regeneration.